times, as yet preventing conclusive evaluation of this new form of treatment (7, e5, e6).

Reconstruction of the conjunctival surface

Excision of a squamous cell carcinoma or a melanoma from the area of the conjunctiva, for example, often causes major surface defects. After complete tumor removal, defects can be covered with an AM (22, 23). Further adjuvant measures to eradicate the tumor, such as radiation or local chemotherapy, can then follow. In cases such as these the AM functions as a basement membrane substitute and aids fast, usually scar-free wound healing by promoting the migration of neighboring conjunctival epithelial cells of the recipient (23) *(Figure 3)*. Thanks to this, AM is more cosmetically acceptable than other methods such as transplantation of oral mucous membranes, and their intrinsic transparency also allows effective post-operative tumor monitoring (22, 23).

Conclusion

AMT is used in acute ophthalmological care, to treat chronic diseases of the surface of the eye, and as the newest development, using tissue engineering, as a biomatrix to treat severe stem cell deficiency of the ocular surface. It provides practicing ophthalmologists with a particularly multifaceted instrument to tackle the challenges posed by disorders of the surface of the eye successfully. Controlled, randomized, multicenter, long-term trials involving large numbers of patients are needed in order to substantiate the clinically relevant potential of AMT in reconstructive surgery of the surface of the eye, which has been documented in many case studies.

Supported by external grants from the Deutsche Ophthalmologische Gesellschaft (the medical scientific association of ophthalmology in Germany), Munich; the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), Bonn, ME 1623/3–1; the Estnische Forschungsgemeinschaft (Estonian Research Foundation), Grant 5832; and the Forschungsförderung AG Trockenes Auge (the German research fund for dry eyes), Berlin, Germany.

KEY MESSAGES

- The amniotic membrane, the innermost layer of the placenta, plays a significant role in reconstructive surgery of the outer eye.
- The amniotic membrane possesses biological properties which promote wound healing in various disorders of the outer eye.
- Cryopreservation preserves the amniotic membrane's biological properties for longer than other methods.
- The main indications of amniotic membrane transplantation are corneal ulceration, covering defects in large conjunctival lesions, and acute chemical burns to the surface of the eye.
- The amniotic membrane is used as a culture substrate and carrier in ex vivo expansion of corneal stem cells, to treat limbal stem cell deficiency.

Conflict of interest statement
The authors declare that no conflict of interest exists.

Manuscript received on 15 December , 2008, revised version accepted on 11 May 2010.

Translated from the original German by Caroline Devitt, MA.

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REVIEW ARTICLE

Amniotic Membrane Transplantation in the Human Eye

Daniel Meller, Mikk Pauklin, Henning Thomasen, Henrike Westekemper, Klaus-Peter Steuhl

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Selective Cell Transplantation Using Bioabsorbable Artificial Polymers as Matrices

By Joseph P. Vacanti, Martin A. Morse, W. Mark Saltzman, Abraham J. Domb, Antonio Perez-Atayde, and Robert Langer

Boston and Cambridge, Massachusetts

● **To date, selective cell transplantation has involved injecting cell suspensions into tissues or the vascular system. This study describes attaching cell preparations to bioerodable artificial polymers in cell culture and then implanting this polymer-cell scaffold into animals. Using standard techniques of cell harvest, single cells and clusters of fetal and adult rat and mouse hepatocytes, pancreatic islet cells, and small intestinal cells have been seeded onto biodegradable polymers of polyglactin 910, polyanhydrides, and polyorthoester. Sixty-five fetuses and 14 adult animals served as donors. One hundred fifteen polymer scaffolds were implanted into 70 recipient animals: 66 seeded with hepatocytes; 23 with intestinal cells and clusters; and 26 with pancreatic islet preparations. The cells remained viable in culture, and in the case of fetal intestine and fetal hepatocytes, appeared to proliferate while on the polymer. After four days in culture, the cell-polymer scaffolds were implanted into host animals, either in the omentum, the interscapular fat pad, or the mesentery. In three cases of fetal intestinal implantation coupled with partial hepatectomy, successful engraftment occurred in the omentum, one forming a visible 6.0 mm cyst. Three cases of hepatocyte implantation, one using adult cells and two using fetal cells, have also engrafted, showing viability of hepatocytes, mitotic figures, and vascularization of the cell mass. To date, no pancreatic islets have survived implantation. This method of cell transplantation, which we have termed "chimeric neomorphogenesis," is an alternative to current methods and requires further study.**

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INDEX WORDS: Transplantation; cell culture; fetal cell transplantation.

SELECTIVE cell transplantation of only those parenchymal elements necessary to replace lost function has been studied as an alternative to whole or partial organ transplantation.[1] In the past, cells have been harvested, dispersed into a suspension, and then innoculated into various tissues. We report attaching parenchymal cells from liver, intestine, and pancreas onto bioerodable artificial polymers and then implanting these polymer-cell scaffolds into animals as a novel method of cell transplantation.

MATERIALS AND METHODS

Polymers

Three synthetic absorbable polymers have been used to fabricate filaments and discs as matrices for cell attachment, growth, and implantation (Fig 1).

Polyglactin. This polymer was developed as absorbable synthetic suture material. It is a 90-10 copolymer of glycolide and lactide and is produced as Vicryl braided absorbable suture (Ethicon Co, Somerville, NJ).[2]

Polyorthoesters. The specific polymer used was 3,9-bis(ethylidene-2,4,8,10-tetraoxaspiro[5.5]) undecane copolymer with *trans*-1,4-cyclohexanedimethanol and 1,6-hexandiol in a molar ratio 2:1:1, respectively (SRI, CA).[3]

Polyanhydride. The specific polymer used was a polyanhydride of bis(1,4-carboxyphenoxy) propane and sebacic acid. It is biocompatible and has been used extensively in drug delivery applications.[3-6]

Polymer Configuration

Small wafer discs or filaments of polyanhydrides and polyorthoesters were fabricated using one of the following methods.

Solvent casting. A solution of the polymer (10% in methylene chloride) was cast on a branching pattern relief structure as a disc 10 mm in diameter for 10 minutes at 25°C using a Carver press. After solvent evaporation, a film 0.5 mm in thickness with an engraved branching pattern on its surface was obtained.

Compression molding. One hundred mg of the polymer was pressed (30,000 psi) into a branching pattern relief structure 10 mm in diameter. 0.5 mm discs were obtained.

Filament drawing. Filaments were drawn from the molten polymer (30 μm in diameter). Small flattened 1.0 cm tufts were used for the experiments.

Polyglactin 910. Multiple fibers of 90-10 copolymer of glycolide and lactide converging to a common base were fashioned from suture material of 0-Vicryl by fraying the braided end of the polymer (Fig 2). These branching fiber clusters were approximately 1.0 cm in height. The individual fibrils were 30 μm in diameter.

Animals

Young adult and fetal Sprague Dawley rats and C57 B1/6 mice (Charles River Labs, Wilmington, MA) were used as cell donors for all experiments. The animals were housed individually, allowed access to food and water ad lib, and maintained at 12 hour light and dark intervals. Anesthesia was obtained with an intraperitoneal injection of pentobarbital (Abbott Labs, North Chicago, IL) at a dose of 0.05 mg/g and supplemented with methoxyflurane (Pitman-

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Supported by the March of Dimes Grant No. 5-543, Basil O'Connor Starter Research Grant, and NIH Grant No. 6M 26698.

Presented at the 18th Annual Meeting of the American Pediatric Surgical Association, Hilton Head Island, South Carolina, May 6-9, 1987.

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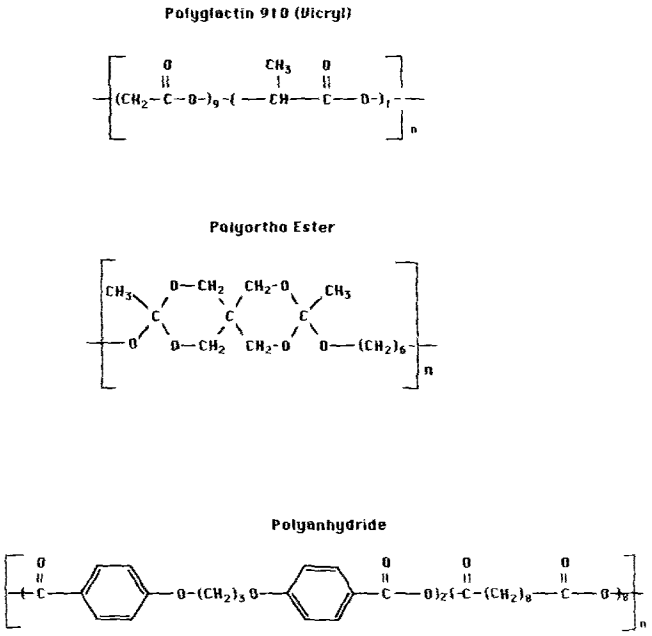


Fig 1. Chemical structure of polymers used for biodegradable cellular matrices. Preparation and configurations are described in text.

Moore, Inc, Washington Crossing, NJ) by cone administration. Fetal animals were harvested at 13, 17, and 20 days gestation and were used as liver, pancreas, and intestinal donors. Young adult animals were used as liver and pancreas donors and as recipients of the cell-scaffold matrices.

Cell Harvest and Cell Culture

Liver. After the induction of anesthesia, the abdomen of young adult animals was shaved, prepped with betadine, and opened using sterile technique. The liver was isolated and after heparinization with 100 U of heparin (Elkins-Sinn, Inc, Cherry Hill, NJ), the portal vein was cannulated with a 23 gauge plastic intravenous cannula (Critikon, Inc, Tampa, FL). The inferior vena cava was transected, and the liver was flushed with 2 to 3 mL of sterile saline. It was then removed from its bed and transferred to a sterile dish where it was perfused with an oxygenated solution of 0.025% collagenase Type II (BCA/Cappel Products, West Chester, PA) by a technique modified from Selgen.[7] After a 20-minute perfusion, the liver was transferred to a sterile hood for cell dispersion and culture.

Fetal animals were harvested by isolating and removing the gravid uterine horns from pregnant animals of the appropriate gestation. The intact uterus with multiple fetuses was transferred in saline to a sterile room, equipped with a dissection microscope. Individual fetuses were opened and the liver, intestine, and pancreas were harvested and pooled. Organs were then transferred to a sterile hood for cell isolation. The tissues were minced, treated with 0.025% Type II collagenase, and dispersed into cell suspensions (Fig 2).

Pancreas. After the induction of anesthesia, the abdomen of young adult animals was shaved, prepped with betadine, and opened in the midline using sterile technique. The common bile duct was isolated, and the pancreas visualized. Two and a half milliliters of 2.0% type II collagenase was infused into the pancreas by injection into the common bile duct using the technique described by Gotoh et al.[8] After five minutes, the pancreas was transferred to a sterile hood for islet cell isolation. Briefly, the tissue was placed into a 25% Ficoll

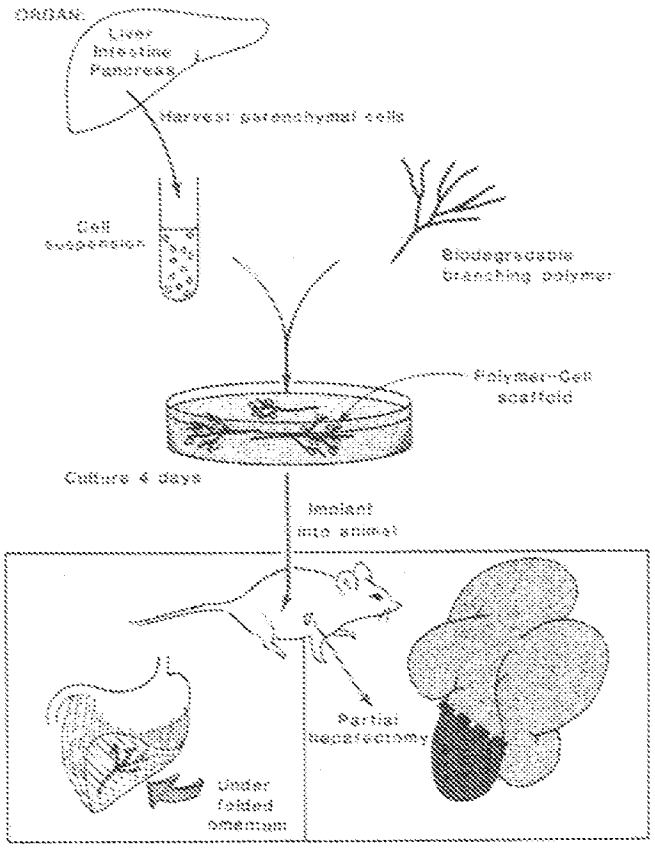


Fig 2. Technique of chimeric neomorphogenesis. Appropriate parenchymal cells are harvested and dispersed. They are seeded onto the polymer matrix in cell culture, where attachment and growth occur. Partial hepatectomy is performed to stimulate growth of the transplant. The polymer-cell scaffold is then implanted into the recipient animal where neovascularization, cell growth, and reabsorption of the polymer matrix occurs.

solution and layered under a discontinuous Ficoll gradient (21%, 11%) and centrifuged at 800 × g for ten minutes. Islets that aggregated at the 21% to 11% interface were washed with cold Hank's solution and centrifuged at 320 × g three times. The islets were resuspended in RPMI 1640 (Gibco, Grand Island, NY) media supplemented with 10% fetal calf serum, and overlaid onto polymer scaffolds. Fetal animals were harvested as donors as described previously.

Intestine. Fetal intestine was obtained as described previously.

Polymer-cell scaffolds and implantation. Cells in suspension were plated onto polymer matrices at 1×10^5 or 1×10^6 cells/mL. They were maintained in Chee's media supplemented with 10% fetal calf serum for 3 to 4 days in a 10% CO_2 environment. Viability of cells on the scaffold immediately preimplantation was assessed by the trypan blue exclusion method. Young adult Sprague Dawley rats were anesthetized, shaved over the operative site, and prepped with betadine.

The polymer-cell scaffold was implanted in one of three sites: (1) the interscapular fat pad; (2) the omentum; and (3) the bowel mesentery (Fig 3).

Most animals underwent a partial hepatectomy to stimulate cell growth. Animals were sacrificed at day 3, 7, or 14 and the implants were examined histologically with hematoxylin and eosin. Polymers without cells served as controls. Polymer-cell scaffolds were exam-

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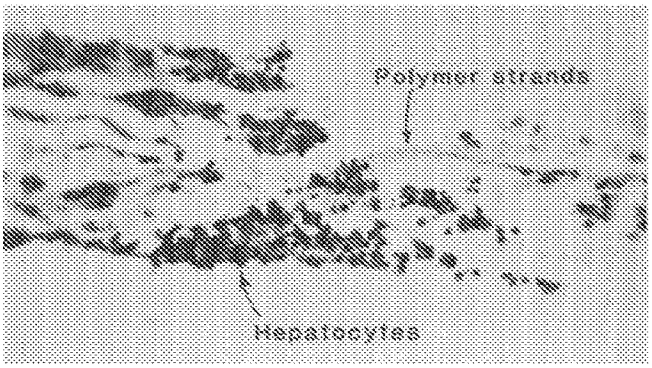


Fig 3. Hepatocytes attached to fibers of polyglactin 910 after four days in culture. Cells vary from healthy to degenerated and necrotic (Hematoxylin and eosin, original magnification ×172).

ined histologically after four days in culture and before implantation to assess cell attachment and viability.

RESULTS

Seventy-nine animals, including 14 adults and 65 fetuses, were used as donors for cell harvest; 115 polymer scaffolds were prepared for implantation. Sixty-six of these scaffolds were seeded with hepatocytes, 23 with intestinal cells and clusters, and 26 with pancreatic islets and cell preparations. Implantation was performed in 70 recipient animals. Fifty-eight were killed at seven days for histologic examination of the implant while three were examined at three days, and nine at 14 days after implantation.

Cell viability on the polymer scaffold at 3 to 4 days in culture varied with the type of polymer material used. Less than 10% of the cells were viable on the polyanhydride discs, whereas 80% of cells cultured on polyorthoester discs and filaments remained viable, and over 90% survived on polyglactin 910 (Fig 3).

Blood vessel ingrowth was noted three days after implantation with all of the polymer types and configurations. In the implanted fiber networks, new blood vessels formed in the interstices between the polymer filaments. The polymer discs showed capillary formation immediately adjacent to the polymer material. This angiogenic response accompanied an inflammatory infiltrate that displayed both an acute phase and a chronic foreign body reaction to the implanted polymers. The intensity of inflammation varied with the polymer type tested: polyanhydride elicited the most severe acute and chronic response although the inflammation surrounding branching fibers of either polyorthoester or polyglactin appeared proportionately greater than the disc configuration because of the greater surface area of exposed foreign material to host.

Histologic examination of liver cell implants in three animals showed evidence of successful engraftment of hepatocytes at seven days. Small clusters of healthy appearing hepatocytes were seen with bile canaliculi between adjacent cell membranes and some areas demonstrated mitotic figures. The cells were surrounded by an inflammatory response and blood vessels coursed around and through the cell clusters. Polymer material was seen immediately adjacent to the cells (Fig 4).

Successful engraftment of intestinal cells and clusters were observed in three animals. Histologic findings were similar to the hepatocyte implants with one exception (Figs 5A-C). On gross examination of the implant at seven days, a cystic structure approximately 6.0 mm in length was found at the implant site (Fig 5A) with polymer fibers splayed within its wall. Microscopic examination revealed well-differentiated intestinal epithelium lining the cavity with mucous and cellular debris within the lumen. One wall of the cyst contained polymer fibers, blood vessels, and inflammatory cells immediately adjacent to the intestinal epithelium (Fig 5B). The other wall included a muscular coating that suggested that the polymer held a small minced piece of fetal intestine as the origin of the cyst that eventually developed. The cyst displayed well-differentiated intestinal epithelium with mucous secreting cells (Fig 5C). Other clusters of intestinal epithelium demonstrated active mitoses.

No viable pancreatic islets or other cell types were found in any of the pancreas implants tested. However, all of the implants were accompanied by an inflammatory and angiogenic response similar to those noted with liver and intestine.

Control polymers implanted without prior cell seed-

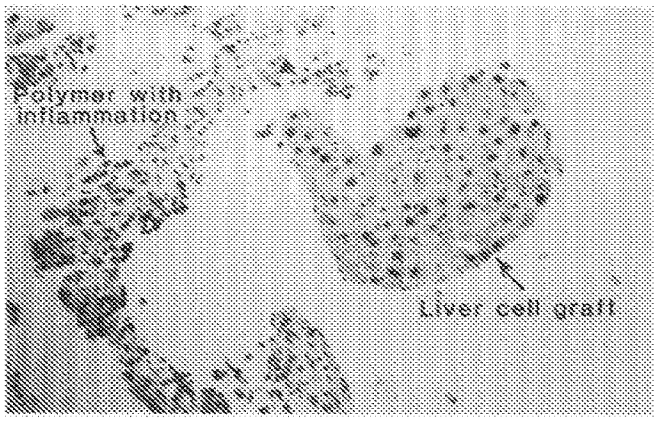


Fig 4. Implant of hepatocytes from adult rat donor into omentum. The polymer-cell implant has been in place for seven days before death. Hepatocytes are healthy and several mitotic figures can be seen. Blood vessels are present in the mass. To the left, an inflammatory infiltrate in the area of the polymer is observed (Hematoxylin and eosin, original magnification, ×172).

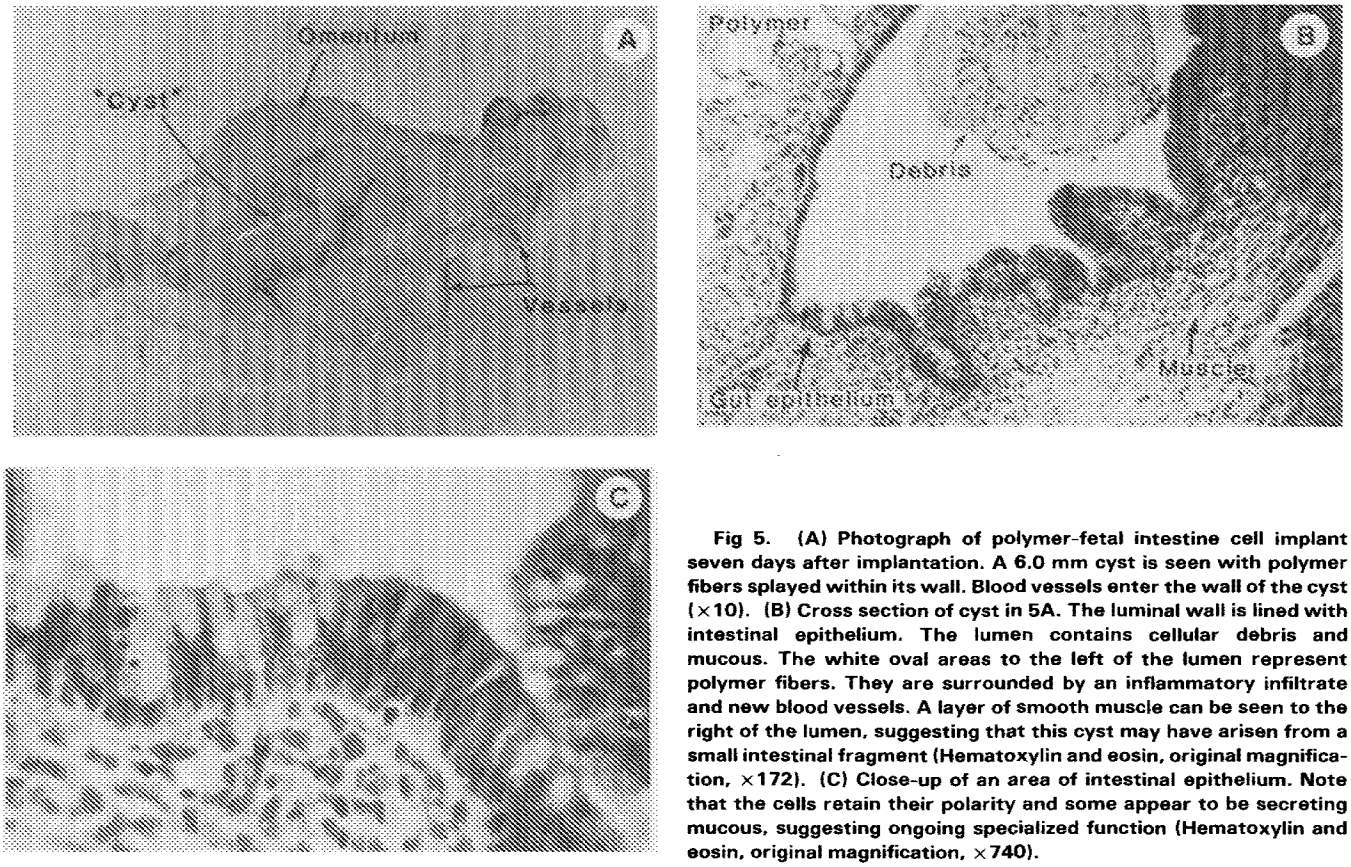


Fig 5. (A) Photograph of polymer-fetal intestine cell implant seven days after implantation. A 6.0 mm cyst is seen with polymer fibers splayed within its wall. Blood vessels enter the wall of the cyst (×10). (B) Cross section of cyst in 5A. The luminal wall is lined with intestinal epithelium. The lumen contains cellular debris and mucous. The white oval areas to the left of the lumen represent polymer fibers. They are surrounded by an inflammatory infiltrate and new blood vessels. A layer of smooth muscle can be seen to the right of the lumen, suggesting that this cyst may have arisen from a small intestinal fragment (Hematoxylin and eosin, original magnification, ×172). (C) Close-up of an area of intestinal epithelium. Note that the cells retain their polarity and some appear to be secreting mucous, suggesting ongoing specialized function (Hematoxylin and eosin, original magnification, ×740).

ing elicited an angiogenic and inflammatory response similar to their counterparts that had been seeded with parenchymal cells and maintained in culture. This suggested that the cells themselves did not play a major role in the inflammation and neovascularization seen.

DISCUSSION

Many diseases of the liver, intestine, and pancreas result in organ failure. This century has seen the development of specific pharmacologic therapy to replace lost function. Insulin replacement for diabetes mellitus is an example.

The emergence of organ transplantation and the science of immunobiology has allowed replacement of the kidney, heart, liver, and other organs. However, as our ability to perform these complex operations has improved, the limitations of the technology have become more evident. For example, in pediatric liver transplantation, donor scarcity has increased as more programs have opened. Only a small number of donors are available in the United States for 800 to 1,000 children in liver failure and those children that undergo transplantation are often so ill by the time a liver is found that the likelihood of success is diminished. The surgery is complex and usually associated with major blood loss. The preservation time is short and, therefore, results in major logistical problems in matching a distant donor with a recipient. For these reasons, the undertaking is expensive and labor intensive, requiring a major investment of resources available only in tertiary care facilities.

Selective cell transplantation of only those parenchymal elements necessary to replace lost function has been proposed as an alternative to whole or partial organ transplantation.[1] It has several attractive features. It avoids major surgery with its attendant blood loss, anesthetic difficulties and complications. It replaces only those cells that supply the needed function and, therefore, problems with passenger leukocytes, antigen presenting cells, and other cell types which may promote the rejection process are avoided. Adding the techniques of cell culture provides another set of tools to aid in the transplantation process. The ability to expand cell numbers with proliferation of cells in culture allows autotransplantation of one's own tissue. Skin equivalents using contracted collagen lattices and epidermal cells have been described.[9,10] Insertion of gene segments, and deletion of antigenic components while the cells are in culture is also possible with current technology.

Islet cell transplantation as an experimental treat-

ment of diabetes mellitus is an area of current research. Although there is evidence of short-term function, long-term results have been less satisfactory.[11,12] Currently, whole organ pancreatic transplantation is the preferred replacement. Hepatocyte injections into the portal circulation have been attempted to support hepatic function. A recent novel approach in which hepatocytes were attached to collagen-coated microcarrier beads prior to injection into the peritoneal cavity demonstrated successful implantation, viability of the implanted hepatocytes, and function. The authors suggested that cell attachment to a maxtrix prior to implantation was an important component of successful engraftment and function.[13]

Our studies of isolating parenchymal cells, attaching them to biodegradable polymer scaffolds in cell culture, and implanting them into hosts are based on several biologic observations:

(1) Every structure in living organisms is in a dynamic state of equilibrium. It undergoes constant renewal, remodeling, and replacement of functional tissue. The degree of change varies from organ to organ and structure to structure.

(2) Structural cells, if placed in a dissociated state, tend toward reforming structure. Their ability to do so depends on the environment in which they are placed and the degree of alteration they have undergone. Examples include capillary endothelial cells that form tubes in vitro under certain conditions[14] and bile duct cells that form tubes under the proper conditions.[15]

(3) Tissue cannot be implanted in volumes >1.0 to 3.0 μL because nutrition is limited by the maximum diffusion distance until angiogenesis occurs.[16]

(4) Cell shape determined by cytoskeletal components and attachment to matrix plays an important role in cell division and differentiated function.[17,18] If dissociated cells are placed into mature tissue as a suspension without cell attachment, they may have a difficult time finding attachment sites, achieving polarity, and functioning because they begin with intrinsic organization. This may limit the total number of implanted cells that can remain viable to organize, proliferate, and function.

We reasoned that if we provided an organized scaffolding to which the cells were already attached, we could increase the total number of implanted cells. Artificial biodegradable polymers were chosen for several reasons. We could engineer configuration, manageability, tensile strength, and rate of degradation to a great degree with a man-made plastic and also might be able to modify the inflammatory response by modifying the material. The polymer material could be coated with other cell types or with attachment factors to increase cell attachment. In addition, we have considerable experience in placing biologically active molecules such as growth factors directly into the polymer, and allowing slow release of these agents in a controlled and predictable way.[19] Finally, by the use of biodegradable matrices, we provide only a temporary scaffold, which eventually is reabsorbed, leaving structural support of the mass to mesenchymal elements supplied by the host, and modified by the implanted cells. Although some of the polymers studied caused somewhat of an inflammatory response, it is likely that further purification will reduce or eliminate this problem. This has already been shown to be the case with ethylene-vinyl acetate[19] and polyanhydrides.[5]

This technology allowed us to design the polymers to meet the biologic needs of the system we wished to create. The configuration of the polymer scaffold must have enough surface area for the cells to be nourished by diffusion while neovascularization occurs. The new blood vessels must interdigitate with the implanted parenchymal elements to continue to support their growth, organization, and function. Polymer discs seeded with a monolayer of cells and branching fiber networks both satisfy these needs. The branching fibers are based on the same principles that nature has used to solve the problem of increasing surface area proportionate to volume increases. All multicellular organisms utilize this repeating branching structure. Branching systems represent communication networks between organs as well as the functional units of individual organs.[20] Seeding this configuration with cells and implanting the structure as fibers allows us to implant large numbers of cells, each of which is exposed to the environment of the host. Therefore, free exchange of nutrients and waste can occur while neovascularization is achieved. If functional, the new three-dimensional mass would be a true chimera of parenchymal elements of the donor, and mesenchymal elements of the recipient. The term "chimeric neomorphogenesis" describes the process that has occurred.

The results of this study demonstrate that cells from liver, intestine, and pancreas can be successfully harvested, and will attach to artificial biodegradable polymers. They will survive in culture in this configuration and can then be implanted into a host in a variety of locations. An inflammatory response that is mediated by both the wound and the nature of the polymer will occur. Successful engraftment of small clusters of hepatocytes and intestinal cells has been demonstrated. However, we do not yet have evidence of cell function in the new environment. Further studies to define the optimal characteristics of the polymer, attachment parameters, growth criteria, and function, need to be performed. Clinical application in diabetes with beta cells of the pancreas, in hepatic failure with

hepatocytes and biliary cells, and in intestinal insufficiency with intestinal epithelium remains the long-term goal.

ACKNOWLEDGMENT

We wish to express our sincere gratitude to Judah Folkman for advice, Dr Jorge Heller for his generous gift of polyorthoester, Deborah Stark for her technical assistance, and Susann Antczak for preparation of this manuscript.

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Discussion

P. Donahoe (Boston): This interesting approach, which uses biodegradable materials as an artificial matrix, has been successfully done in two-dimensional systems by Drs John Burke and Howard Green for the replacement of skin for major burns. Dr Vacanti is attempting this technique in a three-dimensional system. I look forward to further screening of matrix materials in the future. After maintaining dispersed cells from liver, pancreas, and intestine in culture for three days before implantation, small viable implants were found in three of 66 hepatic implants, three of 26 small intestinal implants with a 6 mm cystic structure, and 0 of 23 pancreatic implants reflecting also our own nonsuccess with that particular organ. Though falling far short of providing needed organs for transplantation, which is the author's dream, it does provide us with some promising techniques. I would make a number of suggestions for improving the rate of graft survival. First of all, allogeneic grafts were used and rejection can expect to be high. I would suggest using syngeneic mice to test the hypothesis more strongly. Ontogeny studies should be done in order to determine at which fetal age growth would be allowed. The position of implantation is also vital. We too lost a high percentage of grafts in omentum and in mesentery, as well as the hepatic capsule and in the retroperitoneum, so we turned to the subrenal capsule where structures can grow without being ejected. Can the authors tell us why the polyorthoester was so unsuccessful? Were implantation toxicity studies done before undertaking the present studies? What will be your next experimental designs? We will follow subsequent studies with great interest.

J.P. Vacanti (closing): Syngeneic mice eliminate rejection as a potential mediator of the inflammatory response, which has been significant in some of the polymers we have tested. The time-line studies with different aged animals are very important and I appreciate that point. Our hope is that adult cells can be used with success as can fetal cells. We have evolved the idea that terminal differentiation of cells is probably not very common and that in the appropriate circumstance with the appropriate communication with other cells and matrix, cells can be manipulated and can express genes that they do not usually express. We have come to the conclusion that the matrix component of different tissues probably contributes to the successful engraftment of different cell types. So

which particular location for which particular cell type needs to be worked out. We wonder whether we can take different polymers for which we have a long experience as slow release reservoirs for bioactive molecules and then use them to precondition different tissues with various growth factors and differentiation factors before we do the implant. We might be able to fool the tissue into thinking it is fetal tissue as opposed to adult tissue. The polyanhydrides at the configuration that we used were the least successful and the most toxic and they are very acidic, and it is very difficult to keep up with the acid release in culture and probably in in vivo. More work needs to be done with this particular polymer family. The polyorthoester is reasonably noninflammatory and provided good cell attachment.

Synthetic Polymers Seeded with Chondrocytes Provide a Template for New Cartilage Formation

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Boston and Cambridge, Mass., and Brown Mills, N.J.

A new approach for tissue creation using synthetic biocompatible and biodegradable polymers as templates onto which cells are seeded is presented. This report concerns the generation of homogeneous plates of stable mature cartilage in vivo. The delivery of chondrocytes on synthetic polymers configured to provide a large surface area for cell attachment and thus to allow cell function and survival by diffusion of nutrients has resulted in the creation of macroscopic plates of up to 100 mg of new cartilage subcutaneously in 19 of 21 animals. The approximate dimensions and configuration of the original templates were maintained as new cartilage was formed and the polymers resorbed.

Prior reports have described new cartilage formation using isolated cell suspensions,[1-5] cells attached to a naturally occurring matrix,[6,7] or polypeptides derived from bone.[8,9] Our method differs in that we use *synthetic* biodegradable polymers as templates onto which we seed chondrocytes and then implant into an animal for the purpose of new cartilage formation. We refer to the polymer with its associated chondrocytes as a *cell-polymer construct*. The employment of synthetic rather than naturally occurring polymers allows exact engineering of matrix configuration so that the biophysical limitations of mass transfer are satisfied, allowing formation of large three-dimensional masses of cartilage the size and shape of the original polymer construct. To our knowledge, this is the first report in which stable mature cartilage is created in soft tissue not adjacent to native bone or cartilage.

Materials and Methods

The procedure used to produce the cell-polymer construct is shown briefly in Figure 1 and is described below. Hyalin cartilage was obtained from the articular surfaces of newborn calf shoulders within 6 hours of sacrifice. The shoulders were washed in povidone-iodine 10% solution, and chondrocytes were harvested under sterile conditions using a technique described by Klagsbrun.[10] The isolated cells were quantitated using a hemocytometer, and then the chondrocyte suspension was concentrated to 5×10^7 cells/cc. Next, braided threads of polyglactin 910, a 90:10 copolymer of glycolide and lactide, coated with polyglactin 370 and calcium stearate (0 - 0 Vicryl suture material, Ethicon Co., Somerville, N.J.) were cut into pieces of approximately 17 mm in length. A knot was placed at one end. The suture material was unbraided distal to the knot to expose multiple fibers 14 μm in diameter (Fig. 2).

Using this configuration, we were able to create interfiber distances varying between 0 and 200 μm while keeping the unit intact. We refer to the synthetic bioabsorbable suture material as the *polymer fiber*.

Fifty polymer fibers were placed into tissue culture dishes 35 mm in size (Costar, Cambridge, Mass.) at a density of two polymer units per well. We seeded each of 28 fibers (the experimentals) with 100 μl of the chondrocyte solution and kept 22 polymer fibers free from exposure to chon-

From the Department of Anaesthesiology at Massachusetts General Hospital and Harvard Medical School, the Department of Surgery at Children's Hospital, the Department of Chemical Engineering at Massachusetts Institute of Technology, and the Department of Pathology at the Deborah Heart and Lung Institute. Received for publication June 27, 1990; revised November 8, 1990.

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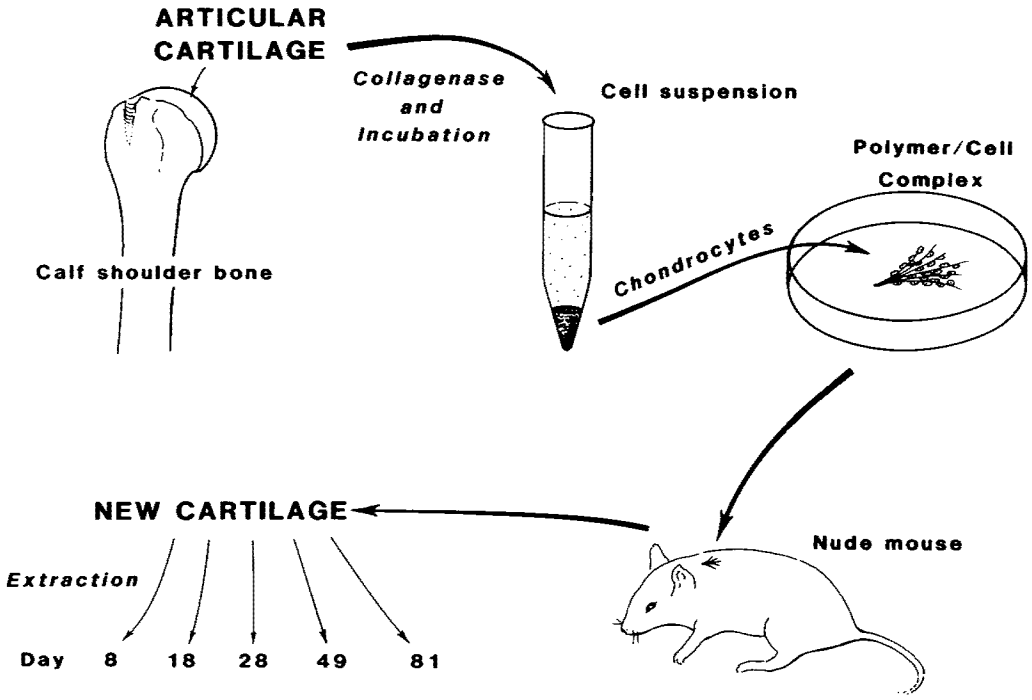


FIG. 1. The implantation of the cell-polymer constructs into nude mice after chondrocyte isolation from articulating surface of calf shoulder and adherence to polymer fibers in vitro. Chondrocytes were harvested from the articular surfaces of newborn calf shoulders as described by Klagsbrun.[10] One hundred microliters of the chondrocyte suspension, concentrated to 5×10^7 cells/cc, was seeded onto braided threads of polyglactin 910 of approximately 17 mm in length and kept in vitro for either 3 or 10 days. Fibers containing chondrocytes were then surgically implanted subcutaneously on the dorsum of 37 male nude mice, 4 to 5 weeks of age, in the midline at the base of the neck. After being implanted for specified periods of times, the implants were excised and fixed in 10% buffered formalin phosphate. To classify the type of collagen present in the specimens, immunohistochemistry was performed using antibodies to bovine collagen types I to IV.

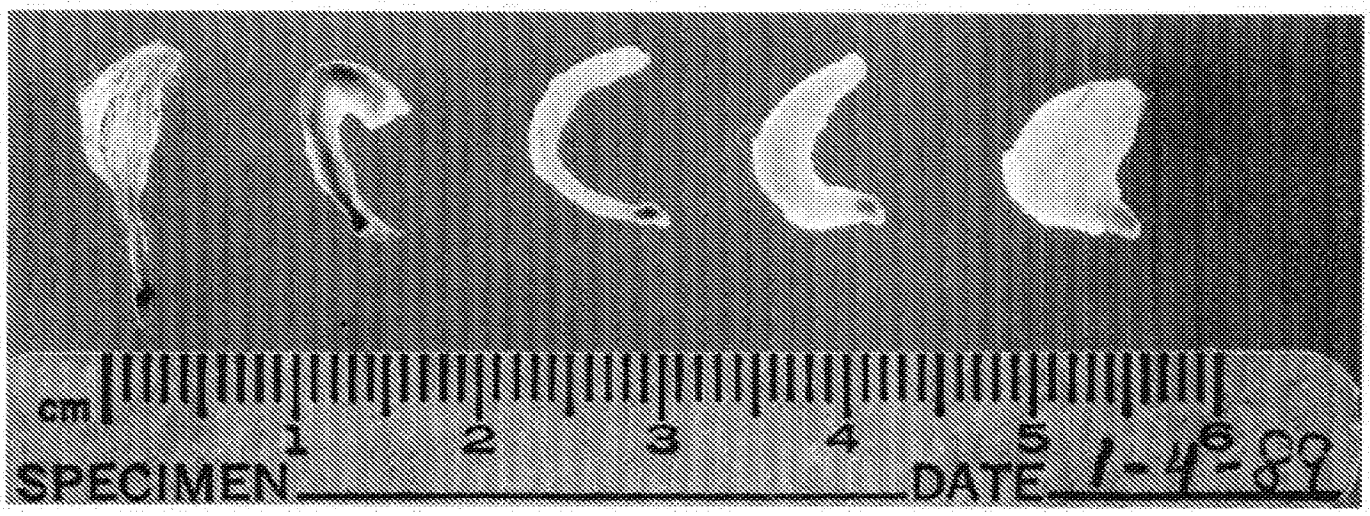


FIG. 2. Photograph of gross experimental specimens. Note the appearance of the polymer fiber prior to seeding with chondrocytes (*far left*). The polymer fiber is an unbraided segment, 17 mm in length, of the suture material 0-0 Vicryl (Ethicon Co., Somerville, N.J.) that has been knotted at one end. To the right of the unseeded polymer fiber are representative specimens of different implants excised (*from left to right*) at days 8, 18, 28, and 49. With the progression of time, the polymer fibers began to dissolve as the cartilage developed into a homogeneous plate of cartilage.

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drocytes (the controls). One of the polymer fibers seeded with chondrocytes was contaminated during our manipulations and was therefore discarded.

After 4 hours, 2 cc of a solution containing Hamm's F-12 culture media (Gibco, Grand Island, N.Y.) and 10% fetal calf serum (Gibco, Grand Island, N.Y.) with L-glutamine (292 μg/cc), penicillin (100 U/cc), streptomycin (100 μg/cc), and ascorbic acid (5 μg/ml) was added to each well. A total of six fibers from each group (experimental and control) were evaluated in vitro for the presence and morphologic appearance of chondrocytes by phase-contrast microscopy and histochemical stains after incubation at 37°C for 3, 6, 11, 18, 21, and 24 days.

Thirty-seven of the remaining fibers (21 experimental and 16 control) were surgically implanted subcutaneously at the base of the neck in the dorsal midline of 37 male nude mice (Athymic *NCr/nude/Sde*, Department of Radiation Medicine, Massachusetts General Hospital) that were 4 to 5 weeks of age. Thirty-two of these implants (16 in experimental group A and 16 in control group A) were performed after incubation for 3 days in vitro, whereas the remaining 5, all in experimental group B, were performed after incubation for 10 days in vitro (Table I). Seven mice with implants (3 in experimental group A with chondrocytes incubated for 3 days, 1 in experimental group B with chondrocytes incubated for 10 days, and 3 in control group A) were sacrificed at each of the following intervals: 8, 18, 28, 49, and 81 days (35 total). Two additional mice, one experimental implanted after 3 days of incubation with chondrocytes and one control, were sacrificed at 168 days.

As another control, 10 mice were injected subcutaneously in the same region with 200-μl cell suspensions containing 5×10^5 chondrocytes without attachment to polymers. Five of these cell suspensions were injected immediately upon isolation of the chondrocytes, whereas the remaining five cell suspensions were injected after incubation of the chondrocytes in vitro for 3 days. These mice were sacrificed at the same intervals of time as the experimental group, and the areas injected were evaluated histologically in the same manner as mentioned earlier for evidence of chondrocytes or cartilage. The data from the two experimental groups were combined for statistical analysis and compared with the control groups using the Fisher's exact test.

After being implanted for specified periods of time, the implants were then excised following a tissue plane that easily separated the implant from the surrounding tissue and were fixed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, N.J.). To classify the type of collagen present in the specimens, immunohistochemistry was performed using antibodies to bovine collagen types I to IV. Hyalin cartilage, being different from other mammalian tissues in that it contains no type I collagen, is one of very few mammalian tissues containing type II collagen. The avidin–biotin-peroxidase technique was used.[11] Chondrocytes in culture readily adhered to the polymer fibers in multiple layers and retained a rounded configuration. This configuration has been correlated with the cell's ability to achieve normal differentiated function.[12] Hematoxylin and eosin staining of the experimental specimens in vitro demonstrated a basophilic matrix at days 18, 21, and 24. Aldehyde fuschin–alcian blue stains[13,14] of the same specimens indicated the presence of sulfated glycosaminoglycans (GAGs). In contrast, histologic evaluation using hematoxylin and eosin stains showed no evidence of chon-

TABLE I
Summary of In Vivo Data

Group	*N*, Total Implants	8 Days	18 Days	28 Days	49 Days	81 Days	168 Days	No. Positive
Experimental A	16 } 21	3	3	3	3	3	1	14 } 19*
Experimental B	5 }	1	1	1	1	1	0	5 }
Control A	16	3	3	3	3	3	1	0
Control B	5 } 10	1	1	1	1	1	0	0 } 0
Control C	5 }	1	1	1	1	1	0	5 }

Note: The left column lists the groups studied in vivo. Experimental groups A and B are very similar and are combined for statistical analysis. Experimental group A consisted of polymer fibers seeded in vitro with chondrocytes 3 days prior to implantation, whereas the polymers in experimental group B were seeded in vitro with chondrocytes 10 days prior to implantation. Control group A consisted of implantation of polymer fibers alone, whereas control groups B and C represented chondrocyte injections not associated with polymer fibers. The chondrocytes in control group B were injected immediately after isolation, whereas those in control group C were incubated for 3 days in vitro prior to injection. The second column lists the total number of implants performed in each group. The next six columns to the right show the numbers of implants excised in each study group at each of six time intervals. The column on the right presents the number of implants in each group in which new cartilage formation was identified. There was new cartilage formation in 19 of 21 experimental animals, whereas no evidence of new cartilage formation was demonstrated in any of the controls.

* Statistical analysis of the results using a Fisher's exact test was significant ($p < 0.01$) when comparing the combined experimental groups with each control group.

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drocytes or chondroitin sulfate in the six in vitro control fibers. All polymer fibers in vitro (control and experimental) began to dissolve by day 27, which is comparable to their anticipated time for dissolution.[15]

Results

The following results were found in specimens that had been surgically implanted into nude mice for increasing periods of time. On gross examination, the experimental polymer fibers were progressively replaced by cartilage, until only cartilage with very little evidence of polymer remained (see Fig. 2). The wet weights of the specimens increased gradually until day 49, after which time they remained stable, averaging 60 to 70 mg. The surface of the newly formed cartilage was smooth in all specimens.

Histologic examination of these specimens (Fig. 3) using hematoxylin and eosin stains revealed evidence of cartilage formation in 19 of the 21 experimental implants (statistically significant from the control groups using the Fisher's exact test, $p < 0.01$), with all specimens having been implanted for at least 28 days appearing very similar to normal human fetal cartilage. A mild inflammatory response, as evidenced by polymorphonuclear leukocytes and giant cells, noted in the day 8 specimens appeared to be resolving in the day 18 and 28 specimens. The resolution of the inflammatory response correlated, at least temporally, with the disappearance of the polymers. Very little evidence of either inflammatory response or polymer remnants was seen in the day 49, 81, and 168 specimens. No lacunae were present in any day 8, 18, 28, or 49 specimens, whereas lacunae were observed in all day 81 and 168 specimens, suggesting the progression from fetal to mature cartilage. A decrease in neovascularization also was noted in these specimens. Aldehyde fuschin–alcian blue staining of these specimens suggested the presence of sulfated glycosaminoglycans (see Fig. 2).

Type III collagen, generally seen only in immature mammalian hyalin cartilage, was demonstrated with immunohistochemistry in the day 49 and earlier specimens but was not seen in later specimens. Type II collagen, remarkable in that it is found almost exclusively in mammalian hyalin cartilage, was not found in early specimens, that is, day 8, 18, or 28 specimens; however, it was demonstrated in specimens implanted 49 days or more. Type I collagen, detectable in most mammalian tissues but not found in mammalian hyalin cartilage, was not detected in any of the specimens. In the 16 polymer fiber control implants not containing chondrocytes, there was no evidence of cartilage formation histologically using the hematoxylin and eosin stain. A mild inflammatory response with polymorphonuclear leukocytes, giant cells, and fibroblasts was noted in these implants at days 8, 18, and 28, after which time no evidence of the implant was found. In the second control group, cartilage formation was not evident in any area injected with a suspension of chondrocytes.

Discussion

This study demonstrates that chondrocytes seeded onto appropriately configured synthetic biodegradable polymers will adhere and perform differentiated function in vitro, as seen by matrix formation. Surgical implantation of this cell-polymer construct into an animal will result in the formation of new cartilage that matures with time, contains type II collagen, and retains the approximate configuration and dimensions of the original template. The new cartilage formed is seen as early as 8 days histologically, and both its wet weight and volume progressed until 49 days, after which time the size of the cartilage formed remained stable, although it matured histologically, as evidenced by the formation of lacunae. We also have shown that the cell-polymer construct is essential in that either injection of free chondrocytes or implantation of the polymer fibers without attached chondrocytes does not result in cartilage formation. Associated with the development of this cartilage formation is a decrease in neovascularization and fibrous tissue formation. This may reflect the production of an angiogenesis inhibitory factor by the newly formed cartilage.[16]

This approach for creating new tissues differs from other approaches in that it utilizes a *synthetic* matrix to which cells adhere, providing structural integrity as the implant engrafts and a new tissue is created.[17] Using synthetic as opposed to naturally occurring substances as matrices allows one the flexibility to alter physical properties and potentially facilitates reproducibility and scale-up. The configuration of the synthetic matrix also can be manipulated to vary the surface area available for cell attachment as well as to optimize the exposure of the attached cells to nutrients. The precise physical characteristics and configuration of the "optimal" polymer design have yet to be determined. The chemical environment sur-

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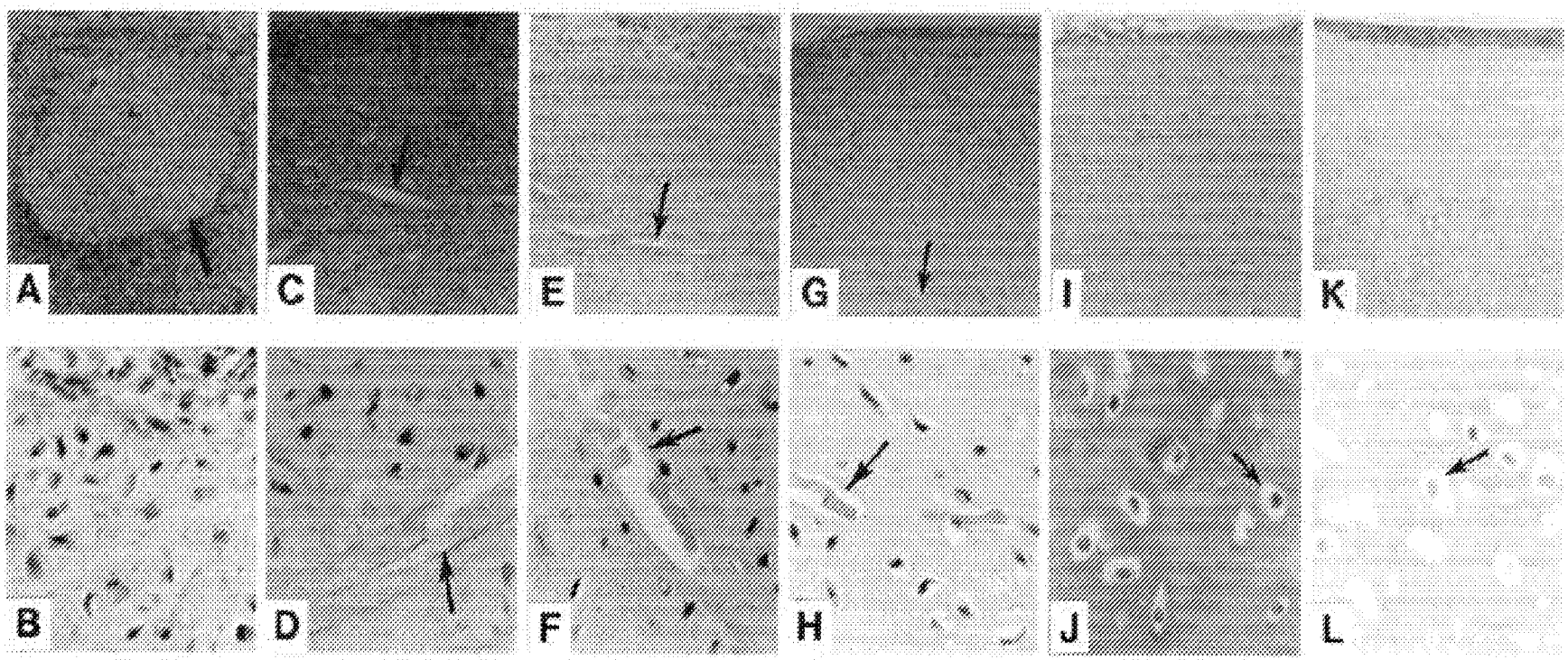


FIG. 3. Hematoxylin and eosin staining of experimental implants excised (*from left to right*) at 8, 18, 28, 49, 81, and 168 days. The upper row is at ×4 magnification, whereas the lower row is magnified ×20. At day 8 (*A*), isolated "nests" of cartilage (*arrow*) were found embedded in fibrous tissue. There was some evidence of a mild inflammatory response consisting of infiltrates of polymorphonuclear leukocytes and giant cells. (*B*) Magnification (×20) of this "nest" of cartilage. Note the presence of polymer fibers (*arrows*) at days 18 (*C, D*) and 28 (*E, F*), after which time we see progressively less evidence of residual fibers. Specimens (*C*) through (*L*) appear to be homogeneous plates of cartilage that mature histologically with time, as evidenced by the following: There is increased deposition of basophilic matrix associated with increasing intercellular distances. Also, the formation of lacunae (*arrows*) in which the chondrocytes are enclosed is observed in the day 81 and 168 specimens (*J* through *L*). This parallels the normal histiogenesis of cartilage. There is no evidence of neovascularization in the day 49 (*G, H*), 81 (*I, J*), and 168 (*K, L*) implants. Aldehyde fuschin–alcian staining was highly suggestive of the presence of chondroitin sulfate in the day 18, 28, 49, 81, and 168 specimens.

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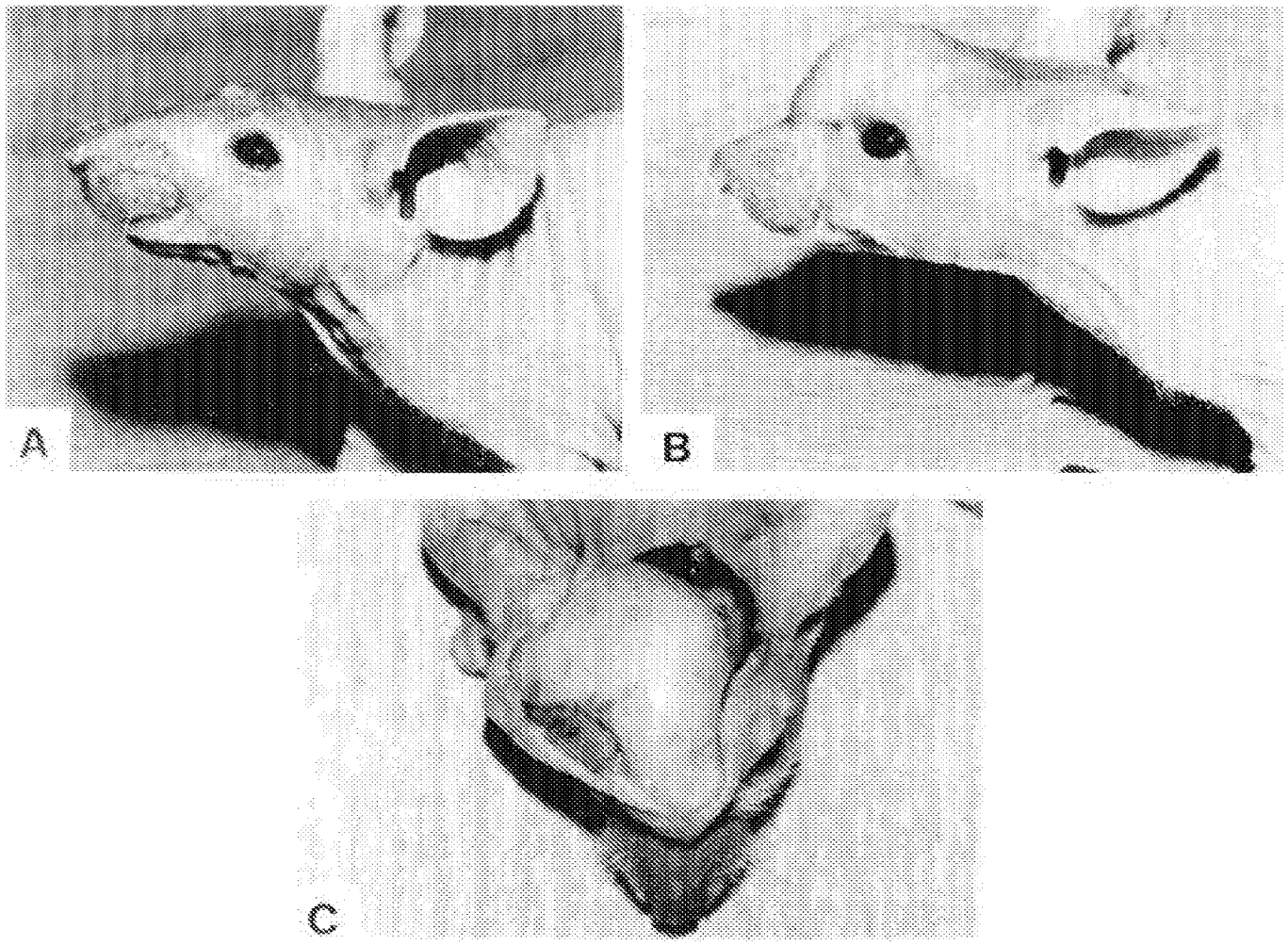


FIG. 4. Cell-polymer constructs composed of a nonwoven mesh of 15-μm-diameter fibers of the synthetic bioabsorbable polymer polyglycolic acid were seeded with 10^7 chondrocytes as in Figure 1. After incubation for 1 week in vitro, they were implanted into nude mice to alter their facial configuration. (*A*) Normal, unaltered nude mouse. (*B*) Nude mouse 7 weeks after receiving a facial implant over the nasal, frontal, and maxillary bones. (*C*) Photograph of the new cartilage created in situ, after sacrifice of the recipient. Histology of this specimen using hematoxylin and eosin staining was consistent with normal fetal bovine cartilage.

rounding a synthetic polymer can be affected in a controlled fashion as the polymer is hydrolyzed. The potential exists to continuously deliver nutrients and hormones that can be incorporated into the polymer structure.[18] Our experiments suggest that the degree of cartilage generated in this manner is such that it may be feasible to alter the physical appearance of the recipients of such implants in a controlled and predictable fashion (Fig. 4). This technology may be useful in plastic and reconstructive surgery as well as in replacing cartilage on articulating surfaces. It is also conceivable that employment of this approach utilizing different cell types may result in the generation of other tissue equivalents.

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ACKNOWLEDGMENTS

We would like to thank Paul Andriesse for his artwork and Jane Landis for her assistance and administrative support in the preparation of this manuscript.

This work was supported by a grant from Neomorphics, Inc. The work describes the results of scientific research and is not related to the development of any product by the authors.

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Syst. Biol. 54(1):91–110, 2005
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ISSN: 1063-5157 print / 1076-836X online
DOI: 10.1080/10635150590906037

Ontogeny Discombobulates Phylogeny: Paedomorphosis and Higher-Level Salamander Relationships

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Abstract.— Evolutionary developmental biology ("evo-devo") has revolutionized evolutionary biology but has had relatively little impact on systematics. We show that similar large-scale developmental changes in distantly related lineages can dramatically mislead phylogenetic analyses based on morphological data. Salamanders are important model systems in many fields of biology and are of special interest in that many species are paedomorphic and thus never complete metamorphosis. A recent study of higher-level salamander phylogeny placed most paedomorphic families in a single clade based on morphological data. Here, we use new molecular and morphological data to show that this result most likely was caused by the misleading effects of paedomorphosis. We also provide a well-supported estimate of higher-level salamander relationships based on combined molecular and morphological data. Many authors have suggested that paedomorphosis may be problematic in studies of salamander phylogeny, but this hypothesis has never been tested with a rigorous phylogenetic analysis. We find that the misleading effects of paedomorphosis on phylogenetic analysis go beyond the sharing of homoplastic larval traits by paedomorphic adults, and the problem therefore is not solved by simply excluding suspected paedomorphic characters. Instead, two additional factors are critically important in causing paedomorphic species to be phylogenetically "misplaced": (1) the absence of clade-specific synapomorphies that develop during metamorphosis in nonpaedomorphic taxa and allow their "correct" placement and (2) parallel adaptive changes associated with the aquatic habitat of the larval stage. Our results suggest that the effects of paedomorphosis on phylogenetic analyses may be complex, difficult to detect, and can lead to results that are both wrong and statistically well supported by parsimony and Bayesian analyses. [Amphibians; development; heterochrony; morphology; ontogeny, paedomorphosis; phylogeny; salamanders.]

The relationship between ontogeny and phylogeny is a recurring theme in developmental, systematic, and evolutionary biology (e.g., Gould, 1977; Alberch et al., 1979; Fink, 1982; Humphries, 1988; Mabee, 1993; McNamara, 1996; Raff, 1996; Futuyma, 1998). But whereas the use of phylogenetic trees to study the evolution of developmental mechanisms has burgeoned (e.g., Carroll et al., 2001; Wilkins, 2001), the importance of ontogenetic data in reconstructing phylogenies has increasingly been questioned (reviewed in Mabee, 2000). Developmental processes may be critically important to systematics if similar, large-scale developmental changes in distantly related lineages can strongly mislead phylogenetic analyses based on adult morphological data. Although this possibility has been suggested in theory (e.g., Emerson and Hastings, 1998), no empirical studies have yet rigorously demonstrated such an outcome. However, some studies have yielded results that might support this hypothesis (e.g., Kluge, 1989; Cunningham and Buss, 1993) or have reconstructed a seemingly misleading tree based on analysis of larval morphology alone (e.g., Wray, 1996).

Salamanders are one of the three major groups of living amphibians and are important model systems in many disciplines of biology (Duellman and Trueb, 1986; Shaffer, 1993; Bruce et al., 2000). Living salamanders include 10 families, 59 genera, and approximately 500 species (Amphibiaweb, 2003). In salamanders, several lineages are thought to have independently become paedomorphic (or neotenic; Gould, 1977; Alberch et al., 1979), a major change in development that may have important consequences for phylogenetic analysis. The assumed primitive life cycle in salamanders begins with an aquatic egg, which hatches into a gilled, aquatic larva, which then passes through metamorphosis to become an air-breathing, land-dwelling adult (Duellman and Trueb, 1986). In the so-called paedomorphic species, the larvae fail to complete metamorphosis and individuals become sexually mature while retaining an overall larval morphology (Fig. 1) and aquatic lifestyle (although the number of putative larval traits may vary considerably among these paedomorphic taxa; Duellman and Trueb, 1986; Rose, 1999). Thus, it has been suggested that a phylogenetic analysis of "adult" morphology in salamanders may group distantly related paedomorphic species based on the shared presence of larval traits rather than their actual relationships (e.g., Hecht and Edwards, 1976; Milner, 1983; Duellman and Trueb, 1986; Good and Wake, 1992; Larson and Dimmick, 1993). However, this hypothesis has never been tested with a rigorous phylogenetic analysis.

Surprisingly, a recent study of higher-level salamander relationships found results that were suggestive of this pattern, but interpreted them very differently. Gao and Shubin (2001) combined original and previously published morphological and molecular data to address relationships among living salamander families and recently described fossil taxa. Their hypothesis, based on combined molecular and morphological data, differed radically from previous phylogenies (e.g., Milner, 1983; Duellman and Trueb, 1986; Larson and Dimmick, 1993) in placing three of the four paedomorphic families in a single clade (Amphiumidae, Proteidae, Sirenidae; families containing only non-transforming species). Gao and Shubin (2001) included many morphological characters that potentially were influenced by paedomorphosis, but they made no attempt to correct for this process analytically and they did not discuss paedomorphosis as a possible explanation for their results.

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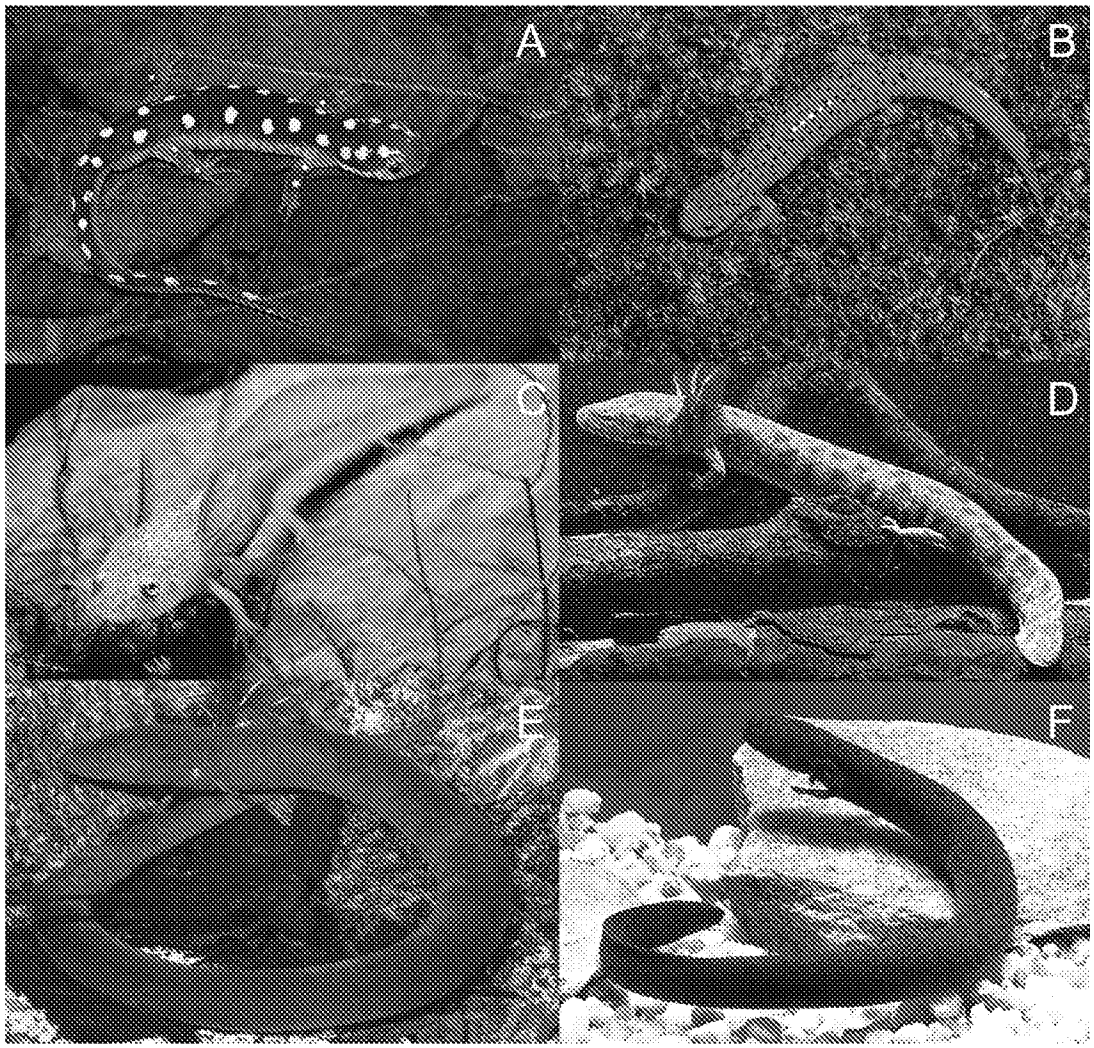


FIGURE 1. Representatives of transforming and paedomorphic salamander families. (A) An adult individual of a metamorphosing species of ambystomatid, *Ambystoma maculatum* (photo by B. Moon). (B) An adult of a metamorphosing plethodontid species, *Pseudotriton ruber* (photo by J. Wiens). (C) A larval ambystomatid of a generally metamorphosing species, *Ambystoma tigrinum* (photo by T. Leenders). (D) An adult of the paedomorphic family Proteidae, *Necturus lewisi* (photo by J. Dermid). (E) An adult of the paedomorphic family Amphiumidae, *Amphiuma means* (photo by W. Van Devender). (F) An adult of the paedomorphic family Sirenidae, *Siren intermedia* (photo by J. Dermid).

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Here we use new morphological and molecular data to show the confounding effects of paedomorphosis on phylogenetic analysis of morphological data in salamanders. More generally, we demonstrate that major developmental changes can mislead higher-level phylogenetic studies based on adult morphology. We show that the effects of paedomorphosis on phylogenetic inference are complex, multifaceted, and often counterintuitive. Finally, we provide an improved phylogenetic framework for studies of salamanders in all biological disciplines.

MATERIALS AND METHODS

Terminology

There is some controversy about definitions of paedomorphosis and of different types of heterochrony in general (e.g., Reilly et al., 1997). We follow standard herpetological usage and refer to salamander species that fail to complete metamorphosis as paedomorphic. We acknowledge, however, that the term "paedomorphic" should generally be applied to individual characters and not whole organisms or taxa, and that our terminology merely represents convenient shorthand. We also acknowledge that retention of juvenile traits can be independent of metamorphosis and may occur in species that lack a larval stage (e.g., Alberch and Alberch, 1981).

We refer to the four families that contain only nontransforming species as "paedomorphic" (Amphiumidae, Cryptobranchidae, Proteidae, Sirenidae) and those three that contain some paedomorphic species and some transforming species as "variable" (Ambystomatidae, Dicamptodontidae, Plethodontidae). Two families that include only a few facultatively paedomorphic species (Hynobiidae, Salamandridae; Duellman and

Trueb, 1986) are not considered variable for the purposes of this study.

Morphological Data and Analysis

Previous morphological studies of salamanders have sampled only a limited number of characters (60 or fewer) and included data only for families rather than individual species (e.g., Milner, 1983; Duellman and Trueb, 1986; Gao and Shubin, 2001). We obtained original morphological data from 32 species of caudates, representing all 10 living families. Representative anurans and caecilians were included as outgroups, based on evidence from morphological (Trueb and Cloutier, 1991) and molecular (Meyer and Zardoya, 2003) data that they are closest relatives to caudates. Species were chosen to represent major groups within families and to match sampling with the molecular data sets as closely as possible (given availability of specimens and tissues). Specimens examined are listed in Appendix 1. Because of our emphasis on relationships among families, our sample sizes within species were small. Morphological analyses including additional species within families yielded similar results to those presented here, suggesting that variation within species should also have little effect at the level of families (Wiens, unpublished).

Most characters were derived from observations of skeletal ($n = 266$) and external variation ($n = 15$). Additional morphological data were obtained from literature studies of vertebral (Edwards, 1976), auditory (Lombard, 1977), and cloacal morphology (Sever, 1991) and literature-based characters of reproduction, larval morphology, and chromosome complement were also included (e.g., Duellman and Trueb, 1986; Crawford and Wake, 1998). Almost all of the characters used by previous authors (e.g., Duellman and Trueb, 1986; Larson and Dimmick, 1993; Gao and Shubin, 2001) were included (with or without modification), with the addition of many new characters. Characters included those that vary within families as well as between families, and many characters were initially gleaned from studies within families (e.g., Tihen, 1958; Wake, 1966; Larsen and Guthrie, 1974; Kraus 1988). Characters are described and listed in Appendix 2. Following previous authors, characters were not excluded a priori because of potential association with paedomorphosis, and sexually mature individuals of all taxa were initially treated as comparable, regardless of whether they were paedomorphic. Alcohol-preserved specimens were prepared as cleared-and-stained skeletal preparations using the method of Dingerkus and Uhler (1977).

Species were coded as terminal units (following Wiens, 1998a), and only presumed "adult" (sexually mature) specimens were used. Multistate characters involving quantitative variation along a single axis (length or extent of ossification of a structure, number of a meristic character) were ordered, and other characters were unordered. We feel strongly that such quantitative traits should be ordered. It makes no sense to assume that similarity in quantitative trait values is important when coding similar taxa with the same character state and then assume that the similarity in trait values is unimportant by giving no order to these states. A parsimony analysis in which these characters are unordered gives similar results to those in which the characters are ordered (results not shown). Specifically, in both analyses of the adult morphology, either all (unordered) or most (ordered) of the paedomorphic salamander taxa form a single weakly supported clade.

Binary characters that showed variation within species were coded using the frequency step-matrix approach (Wiens, 1995, 1999), but one polymorphic unordered multistate character was coded using the polymorphic method (Wiens, 1995, 1999). Under the frequency step-matrix approach, taxa with different trait frequencies are each given a different character state, and the costs of changes between these states are weighted based on the differences in frequencies. Weighting is implemented using a step matrix. This frequency method performs as well as or better than other coding methods in simulation analyses and congruence studies of morphological data (reviewed in Wiens, 1999).

In cases where an individual exhibited different states on different sides (asymmetry), each side was counted separately (as one half of an individual) in calculations of the frequency for that species. This convention makes sense biologically in that individuals that exhibit bilateral variation presumably have intermediate conditions for whatever genetic and/or ontogenetic mechanisms control the expression of the trait. The morphological data matrix is available on the website of the journal.

Although use of larval morphology might be seen as a panacea for problems of paedomorphosis in adult salamander morphology, variation in larval morphology appears to be relatively limited in salamanders, with only two informative characters in this study (numbers 323 and 324 in Appendix 2, number of larval gill slits and presence of larval balancer). These characters were not included in the analyses of adult morphology, and they have little impact on the results if added.

The four fossil taxa (*Karaurus, Laccotriton, Sinerpeton, Valdotriton*) included in the analyses of Gao and Shubin (2001) were included in a set of analyses in this study, based on data from the literature. However, analyses of the morphological and combined data showed that these taxa are of highly ambiguous placement among salamander families and have little impact on relationships estimated for the extant taxa. They are not considered further here.

The most parsimonious tree was sought using a heuristic search with 50 random-taxon-addition replicates, using PAUP* version 4.0b10 (Swofford, 2002). Support for individual nodes was evaluated using non-parametric bootstrapping (Felsenstein, 1985), with 200 bootstrap pseudoreplicates per analysis, each with five random-taxon-addition replicates. Bootstrap values $\geq 70\%$ were considered to be strongly supported, following Hillis and Bull (1993; but see their caveats).

A set of phylogenetic analyses was performed in which 30 putative paedomorphic characters were excluded

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from the morphological data matrix. These characters were identified as "paedomorphic" based on the presence of states in both larvae of transforming species (Wilder, 1925; Bonebrake and Brandon, 1971; Worthington and Wake, 1971; Reilly, 1986, 1987; Rose, 1999) and adults of nontransforming species. Rigorous determination of a trait as paedomorphic depends on the species, its phylogenetic relationships, and the ontogeny of its close relatives (Fink, 1982). In other words, whether or not a given character is "paedomorphic" may depend on which portion of the tree is being considered. However, we followed previous authors in designating and deleting a set of paedomorphic characters prior to the phylogenetic analysis to evaluate the effects of this practice on the results. This matrix will also be available on the website of the journal.

Bayesian analysis of the morphological data was also performed, using the maximum likelihood model for discrete morphological character data (Markov k or Mk) developed by Lewis (2001), implemented with MrBayes versions 3.03 and 3.04 (Huelsenbeck and Ronquist, 2001). All Bayesian analyses used four chains and default priors (i.e., specific values of model parameters were not defined a priori). Furthermore, for all Bayesian analyses, log-likelihood scores were examined for equilibrium over time (using visual examination of plots generated using the *sump* command of MrBayes), and those trees generated before achieving stationarity (or the first 100,000 generations, if stationarity was reached before this point) were discarded as "burn-in." Clades with Bayesian posterior probabilities of $\geq$95% were considered to be strongly supported (e.g., Alfaro et al., 2003).

Because available versions of MrBayes do not allow for use of step matrices or large numbers of character states per character, it was not possible to use frequency coding or gap-weighting. Polymorphic characters were coded using the majority approach (for all frequencies >50% or <50%), which should approximate the frequency method and shares many of the same advantages (Wiens, 1995, 1999). Species with a trait frequency of 50% for a given character were coded using the polymorphic method (Wiens, 1999). Gap-weighted characters (numbers 232, 237, and 326) were recoded to have a maximum of 5 states per character and were ordered.

In order to find the best-fitting model of evolution for the Bayesian analysis of the morphological data, we compared two models using Bayes factors (following Nylander et al., 2004). The first was the Mk model assuming equal rates of change among characters. The second model used the gamma distribution to incorporate unequal rates among characters (Mk+Γ). We performed a Bayesian analysis of the adult morphological data under each model, with each analysis using 2.0×10^6 generations sampled every 100 generations. We then obtained the harmonic mean of the likelihoods of the post-burn-in trees from each analysis using the *sump* command in MrBayes. The Bayes factor (B_{10}) was calculated as the ratio of the model likelihoods of the two models under consideration. Values of $2\log_e(B_{10})$ were calculated (the difference in the harmonic means of the log likelihoods of the two models multiplied by two) and values >10 were considered to be very strong evidence favoring one model over the other (Kass and Raftery, 1995). These analyses strongly favored the Mk+Γ model (mean likelihood = −3797.02) over the Mk model (mean likelihood = −3876.73), with a Bayes factor of 159.42.

After choosing the best-fitting model, we performed a replicate Bayesian analysis of the morphological data, using 2.0×10^6 generations. The two analyses converged on very similar topologies and levels of branch support.

Molecular Data and Analysis

The nuclear ribosomal RNA sequence data of Larson and Dimmick (1993), also used by Gao and Shubin (2001), were analyzed. Nine additional taxa included by Larson (1991) but not Larson and Dimmick (1993) were added to increase taxon sampling in the ribosomal data set. Inferred insertion-deletion events were coded as binary characters separate from the nucleotide sequence characters and gaps within sequences were coded as missing data (Simmons and Ochoterena, 2000). The rRNA data set includes a few characters from the small subunit (the first 13 in the data matrix) and all other characters are from the large subunit.

New sequences of the nuclear recombination-activating gene 1 (RAG-1) were obtained from 32 salamander species and three outgroup species (an additional anuran outgroup sequence, *Xenopus laevis*, was obtained from Genbank, accession number L19324; Greenhalgh et al., 1993). Specimens sequenced are listed in Appendix 3. Genomic DNA was extracted from frozen or ethanol-preserved tissues using standard techniques. Targeted sequences were amplified using the polymerase chain reaction (PCR) in MJ-research thermocyclers using previously developed primers (Greenhalgh et al., 1993; Ventakesh et al., 2001) and new primers designed for this study. Primers and PCR conditions are available on request from P.T.C. Overlapping PCR products were generated with high-fidelity polymerases, purified, and either cycle-sequenced using ABI Prism Big Dye 3.0/3.1 chemistry and an ABI 377 automated sequencer, or with a minimum of two cloned PCR products per amplification, using Thermosequenase chemistry (USB) on a LiCor4200L automated sequencer. Sequences were edited and aligned using Sequencher 3.1.1, and are deposited in Genbank (accession numbers AY650117 to AY650148; see Appendix 3).

RAG-1 sequences were analyzed separately and together with the ribosomal sequences using equally weighted parsimony and Bayesian methods. Combined analysis of the RAG-1 and ribosomal sequences introduced some potential problems. For a few taxa, only RAG-1 and morphological data were available, and therefore ribosomal characters were coded as missing data. For several other taxa, RAG-1 and ribosomal data were available for closely related but

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non-identical species and were combined (e.g., the genus *Desmognathus* is represented by RAG-1 data for one species and by ribosomal data for another), given that our focus is on higher-level relationships of salamanders. The following taxa were combined (species sequenced for RAG-1 listed, those sequenced for ribosomal genes in parentheses): *Ambystoma gracile (tigrinum), Ambystoma opacum (maculatum), Amphiuma pholeter (tridactylum), Aneides lugubris (flavipunctatus), Batrachoseps major (attenuatus), Bolitoglossa helmrichi (subpalmata), Desmognathus quadramaculatus (ochrophaeus), Dicamptodon tenebrosus (aterrimus), Plethodon elongatus (dunni), Pseudotriton ruber (montanus), Rhyacotriton variegatus (kezeri), Salamandra salamandra (Pleurodeles waltl)*, and *Dermophis mexicanus (Typhlonectes compressicauda)*. As an alternative approach, we could have treated each of these nonidentical taxa as separate units in the phylogenetic analysis, coding all 22 taxa with missing data for either RAG-1 or the ribosomal genes. This would have greatly increased the amount of missing data in the matrix, which may dramatically decrease the ability of added characters to improve phylogenetic accuracy (Wiens, 1998b). Furthermore, because of the close relationships of these pairs of species and the incompleteness of the added taxa, the increase in number of taxa might be unlikely to improve accuracy by breaking up long branches (Wiens, 2003). As a third option, we could have simply deleted all taxa that lacked matching data for all data sets. However, this approach would eliminate many critical taxa and would potentially create problems of long-branch attraction among the taxa that were included. In summary, we see our approach as the most reasonable among the alternatives that are possible given the existing data.

Choosing a combination of models (e.g., Jukes-Cantor, general time reversible) and partitioning strategies (e.g., a different model for each gene) for Bayesian analysis is a complex and unresolved issue. Although Bayesian model selection may have important advantages relative to use of likelihood-ratio tests (Nylander et al., 2004), thoroughly testing each possible combination of models and partitioning strategies based on Bayesian analysis would be difficult (i.e., given the many models that could be applied to each data set and all the possible combinations of these models for the combined analysis). We therefore used a "mixed" strategy, in which we used hierarchical likelihood-ratio tests (implemented in Modeltest version 3.06; Posada and Crandall, 1998) to pick reasonable models for the RAG-1 and rRNA data sets separately and then used Bayes factors to refine our model choice and select the best partitioning strategy.

Modeltest selected the GTR+I+Γ model for the RAG-1 data (general time reversible [Rodriguez et al., 1990] with a proportion of sites invariable [Gu et al., 1995] and rates at other sites varying according to a gamma distribution [Yang, 1994]) and the HKY model for the ribosomal data (Hasegawa et al., 1985). Therefore, we did not consider simpler models for these data in subsequent analyses.

We then used Bayesian analyses to address the following questions: (1) Does a single model (GTR+I+Γ) and linked model parameters best fit the combined RAG-1 and ribosomal data rather than separate models and model parameters? (2) Does a more complex model (GTR+Γ) better fit the ribosomal sequences than HKY (given that only parsimony informative sites are included in this data set, a bias which may limit the efficacy of Modeltest and precludes consideration of invariant sites)? (3) Should the RAG-1 data set be divided into separate partitions for each codon position? (4) Does the use of different model parameters for each codon position in the RAG-1 data obviate the need to include parameters for among-site rate variation and invariant sites (I+Γ parameters)? (5) Is a parameter for among-site rate variation necessary for the gap characters (i.e., testing whether Mk or Mk+Γ is more appropriate)?

To address these questions, we tested a total of 11 modeling strategies (MS; Tables 1 and 2) for the combined molecular data. For each, we ran a Bayesian analysis using 1.0×10^6 generations sampled every 100 generations, with four chains and default priors. All of these analyses went to stationarity very quickly (<100, 000 generations) and all produced topologies that were identical or very similar to each other. As described above (see *Morphological Data and Analysis*), we then obtained the harmonic mean of the likelihoods from each analysis and calculated Bayes factors to compare each combination of models and partitions. The results of these analyses suggest that strategy MS4 has the highest likelihood (Table 1) and is very strongly favored over all others based on comparison of Bayes factors (Table 2). This strategy uses unlinked model parameters between the RAG-1 and ribosomal sequences, a complex model for the ribosomal sequences (GTR+Γ), separate partitions for each codon position in RAG-1 (each incorporating among-site rate variation and invariant sites), and among-site rate variation for the gap characters. Using this strategy, we then

TABLE 1. Different modeling strategies used for Bayesian analysis of the combined molecular data, including the number of free parameters and the harmonic mean of the likelihoods from a preliminary analysis. Modeling strategy 4 (MS4) has the highest likelihood. MS4 is also the most parameter-rich model, but the number of parameters alone does not necessarily determine which strategy has the highest likelihood (e.g., compare MS2 and MS6). L = parameters are linked. 3 = separate partition and model parameters for each codon position.

	Molecular partitions				
Model	RAG-1	rRNA sequences	rRNA gaps	No. free parameters	Mean likelihood
MS1	GTR+I+Γ	HKY	Mk+Γ	15	−16,244.44
MS2	L-GTR+I+Γ	L-GTR+I+Γ	Mk+Γ	11	−16,361.33
MS3	GTR+I+Γ	GTR+Γ	Mk+Γ	20	−16,246.10
MS4	3-GTR+I+Γ	GTR+Γ	Mk+Γ	40	−15,910.41
MS5	3-GTR+I+Γ	HKY	Mk+Γ	35	−15,925.43
MS6	3-GTR	GTR+Γ	Mk+Γ	34	−17,156.17
MS7	3-GTR	HKY	Mk+Γ	29	−17158.30
MS8	3-GTR+I+Γ	GTR+Γ	Mk	39	−15,918.41
MS9	3-GTR+I+Γ	HKY	Mk	34	−15928.37
MS10	GTR+I+Γ	GTR+Γ	Mk	19	−16256.95
MS11	GTR+I+Γ	HKY	Mk	14	−16253.90

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TABLE 2. Comparison among all modeling strategies (MS1 to MS11) for the combined molecular data using Bayes factors, following Nylander et al. (2004). Each value represents 2 $\log_e(B_{10})$, with negative values indicating support for the column model over the row model. Model MS4 is strongly favored overall.

	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9	MS10	MS11
MS1	0										
MS2	−233.78	0									
MS3	−3.32	230.46	0								
MS4	668.06	901.84	671.38	0							
MS5	638.02	871.80	641.34	−30.04	0						
MS6	−1823.46	−1589.68	−1820.14	−2491.52	−2461.48	0					
MS7	−1827.72	−1593.94	−1824.40	−2495.78	−2465.74	−4.16	0				
MS8	652.06	885.84	655.38	−16.00	14.04	2475.52	2479.78	0			
MS9	632.14	865.92	635.46	−35.92	−5.88	2455.60	2459.86	−19.92	0		
MS10	−25.02	208.76	−21.70	−693.08	−663.04	1798.44	1802.70	−677.08	657.16	0	
MS11	−18.92	214.86	−15.60	−686.98	−656.94	1804.54	1808.80	−670.98	−651.06	6.10	0

performed two replicate analyses, each using 2.0×10^6 generations, which converged on identical topologies.

Combined Analysis and Ameliorating the Effects of Paedomorphosis

We also performed combined analyses of the molecular and morphological data sets using parsimony and Bayesian methods. Most species in the morphological data set were perfectly matched to species in either the RAG-1 and/or ribosomal RNA data sets, but with some exceptions. We used the morphologically primitive and generalized *Discoglossus jeannae* in place of the highly modified *Xenopus laevis* (see Cannatella and de Sa, 1993: fig. 5), given that the goal of including these taxa is to estimate the ancestral condition for morphological characters in anurans. We excluded two paedomorphic taxa not represented in any of the molecular data sets (*Ambystoma taylori, Dicamptodon copei*). Because of specimen availability we also used the following species in the morphological data set to represent their counterparts in the molecular data sets (species in RAG-1 data set in parentheses): *Ascaphus truei (montanus), Dicamptodon ensatus* (*tenebrosus*), *Necturus maculosus (beyeri), Pseudoeurycea werleri* (*rex*), *Rhyacotriton olympicus (variegatus),* and *Taricha torosa (rivularis). Necturus maculosus* was used to represent *Necturus* morphology in the combined analysis because it is more well characterized for literature-based morphological characters than *N. alabamensis*, and the redundant *N. alabamensis* was deleted.

Our results show that paedomorphosis has a strong misleading impact on phylogenetic analysis of morphology. Thus, simply combining the molecular and morphological data without taking into account the effects of paedomorphosis is potentially problematic. There are at least three potential solutions that might be applied: First, to exclude characters that are seemingly affected by paedomorphosis (e.g., Duellman and Trueb, 1986), second, to exclude paedomorphic taxa (i.e., those taxa that fail to complete metamorphosis), third, to code the adult morphology of paedomorphic species as unknown, given the assumption that the adults of paedomorphic species are not at a comparable ontogenetic stage to adults of transforming species.

All three approaches have disadvantages. Exclusion of paedomorphic characters requires that these characters be identified, which may be difficult prior to the phylogenetic analysis (see *Morphological Data and Analysis*), and our results suggest that excluding suspected paedomorphic characters a priori is not an effective solution. Exclusion of paedomorphic taxa leaves the phylogenetic position of these lineages unresolved and is therefore also undesirable. Coding paedomorphic taxa as unknown may be overly conservative in that many characters that are not affected by paedomorphosis may also be treated as unknown. Nevertheless, we consider this third approach to be the most reasonable, in that it minimizes the potential impact of paedomorphosis on the combined analysis but still allows the position of the paedomorphic taxa to be addressed (albeit with limited data).

In the combined analyses, all characters of adult morphology (characters 1 to 317) were coded as unknown for the nontransforming taxa (amphiumids, cryptobranchids, proteids, sirenids, and the plethodontid *Eurycea neotenes*). Thus, the position of these taxa was addressed only by the molecular data and by the reproductive, larval, and chromosomal characters.

In parsimony analyses, molecular characters were weighted equally with respect to each other and were treated as equivalent to fixed morphological characters (analysis based on successive weighting gave very similar results; Wiens, unpublished). In Bayesian analyses, we used the models and partitioning strategies that were selected in the separate analyses of the molecular (MS4; Table 1) and morphological data (Mk+Γ). Model parameters in the molecular and morphological data sets were unlinked. A limited set of analyses suggests that the best modeling strategies for the molecular and morphological data in the combined analysis is the same as for these data sets when analyzed separately.

For the sake of completeness, we also performed combined-data parsimony and Bayesian analyses in which (1) all data were included for all taxa (i.e., paedomorphic taxa did not have their adult morphological data replaced with question marks); (2) the 30 paedomorphic characters were excluded but all other data were included; and (3) the 30 paedomorphic characters were

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excluded and the adult morphological data of paedomorphic taxa were replaced with question marks. These analyses gave results that are the same as or almost identical to those of the combined parsimony and Bayesian analyses (respectively) in which all the morphological characters are included and paedomorphic adults are coded as unknown. These results therefore are not shown.

Results and Discussion

Do the Paedomorphic Salamander Families Really Form a Clade?

Our analyses of morphological and molecular data suggest that placement of the paedomorphic families in a single clade reflects the effects of paedomorphosis rather than phylogenetic history. We performed an extensive analysis of adult morphology, including more than 300 characters for 32 representative salamander species. As in previous studies (Larson and Dimmick, 1993; Gao and Shubin, 2001), characters that potentially were influenced by paedomorphosis were not excluded. The resulting parsimony and Bayesian trees (Figs. 2, 3) are similar to the phylogeny postulated by Gao and Shubin (2001) in placing three of the four paedomorphic families of salamanders in a single clade (Amphiumidae, Proteidae, Sirenidae; note that salamander monophyly is not supported in the parsimony analysis). Paedomorphic families are those containing only nonmetamorphosing species. Three other families (Ambystomatidae, Dicamptodontidae, Plethodontidae) contain genera in which some species are paedomorphic and others are not. For each of these three variable families, one paedomorphic species was included along with a nonpaedomorphic (transforming) congener. In the resulting parsimony and Bayesian trees, paedomorphic species of these three families are placed in the clade with other paedomorphic species and families, rather than with their transforming congeners (Figs. 2, 3). This result suggests that paedomorphic species are placed in this clade because of their similar developmental modes, rather than their actual phylogenetic relationships.

Two additional lines of evidence confirm that placement of most paedomorphic species in a single clade likely is incorrect. First, combined analyses of data from

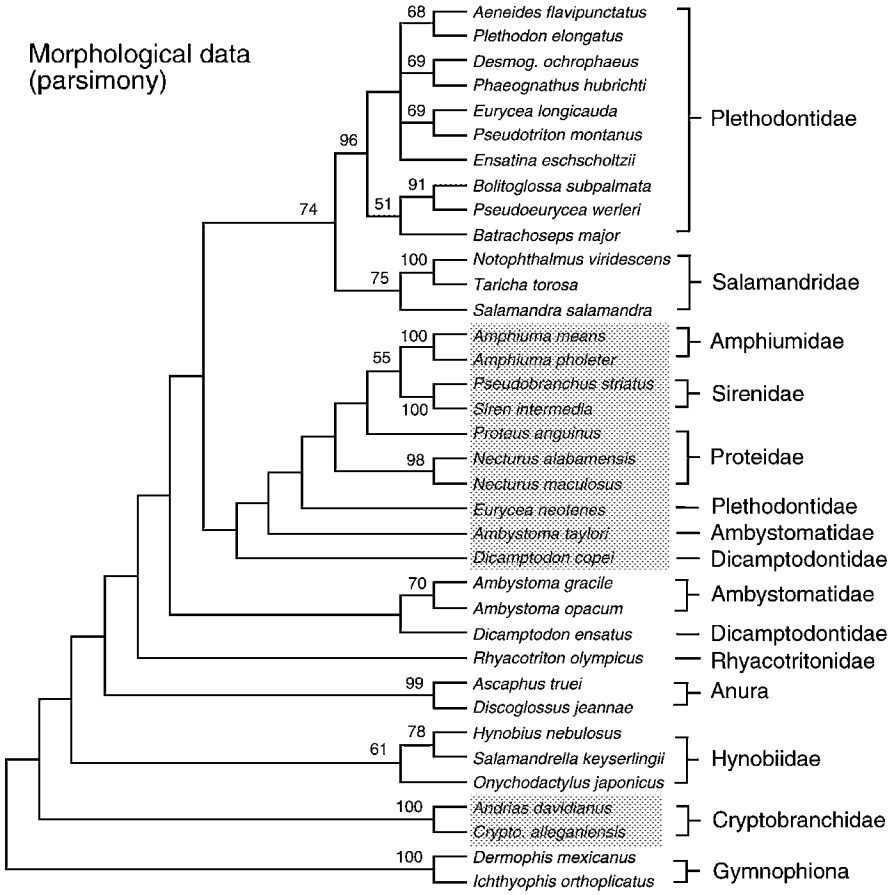


FIGURE 2. Phylogenetic relationships of salamanders based on parsimony analysis of adult morphological characters (paedomorphic taxa shaded). Note the placement of most paedomorphic families and species in a single clade. Numbers above branches indicate bootstrap values ≥50% (values <50% not shown). There are 317 characters (298 parsimony-informative [PI]) and four shortest trees (strict consensus shown), with length = 1090, consistency index (CI) = 0.3271 (excluding uninformative characters), and retention index (RI) = 0.6240. For all figures: *Crypto.* = *Cryptobranchus; Desmog.* = *Desmognathus*.

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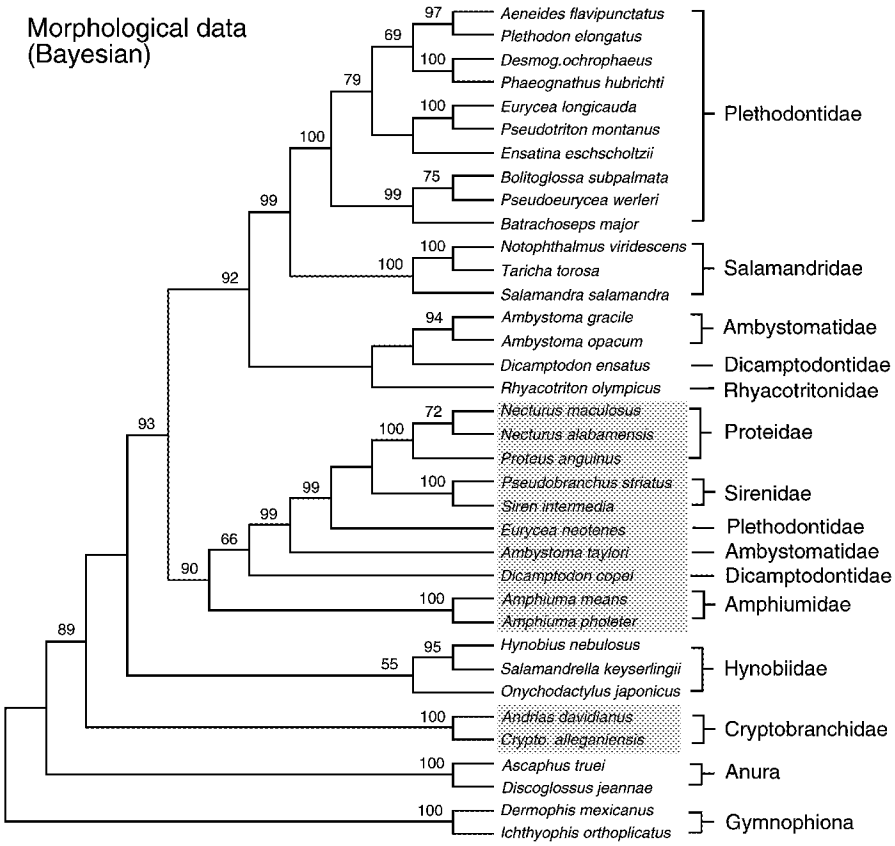


FIGURE 3. Phylogenetic relationships of salamanders based on Bayesian analysis of adult morphological characters (paedomorphic taxa shaded). Note the placement of most paedomorphic families and species in a single clade. Numbers above branches indicate posterior probability values ≥50% (values <50% not shown).

the nuclear RAG-1 gene with nuclear ribosomal RNA sequences strongly refute the placement of the three paedomorphic families in a single clade (Fig. 4). Instead, these results support a more conventional hypothesis of salamander relationships, placing cryptobranchids, hynobiids, and sirenids at the base of the salamander tree, with none of the paedomorphic families as sister taxa (e.g., Milner, 1983; Duellman and Trueb, 1986; Larson and Dimmick, 1993). Almost all major nodes in this phylogeny are strongly supported by Bayesian posterior probabilities and (in most cases) parsimony bootstrap values, and these molecular data sets should be unaffected by paedomorphosis. The failure of these three paedomorphic families to cluster together also is shown in separate analyses of these two nuclear gene regions (Larson and Dimmick, 1993; Wiens, unpublished), and in analyses of mitochondrial DNA sequences using a more limited sampling of taxa (Hedges and Maxson, 1993; Hay et al., 1995).

Second, studies of allozyme data within ambystomatids (Shaffer, 1993), dicamptodontids (Good, 1989), and plethodontids (Chippindale et al., 2000) show relatively little genetic divergence between the paedomorphic species and their transforming congeners, a result confirmed also by analyses of mitochondrial DNA sequences in plethodontids (Chippindale et al., 2000). This limited genetic divergence supports the idea that these paedomorphic species are closely related to congeneric transforming species, and not paedomorphic species in other families.

It is obvious that previous authors were able to correctly assign paedomorphic species to these variable families and genera, based on various types of intrinsic and extrinsic evidence (e.g., biogeography, similarity between larvae of transforming species and paedomorphic adults). Our results show that the misleading effects of paedomorphosis can outweigh the correct phylogenetic signal in the morphological data for these taxa.

How Does Paedomorphosis Influence Phylogenetic Analysis?

The placement of distantly related paedomorphic species in a single clade shows that major changes in development (such as loss of metamorphosis) can lead to grossly incorrect phylogenetic results, even at higher taxonomic levels. However, the effects of paedomorphosis on salamander morphology and phylogenetic analysis are multifaceted and complex.

If the adult morphology of paedomorphic species simply reflected similar truncations of a shared ontogenetic trajectory we might expect all paedomorphic species to be placed in a single, strongly supported clade based

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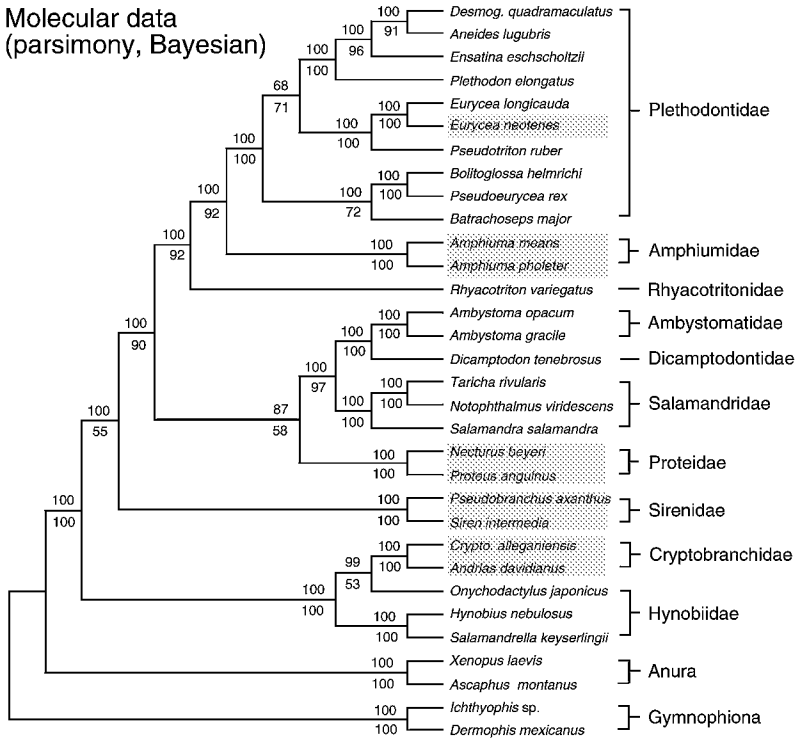


FIGURE 4. Salamander phylogeny based on parsimony and Bayesian analyses of combined RAG-1 and nuclear ribosomal data (paedomorphic taxa shaded). Numbers above branches indicate Bayesian probabilities, below are parsimony bootstraps (≥50%). For some taxa, RAG-1 and ribosomal data were not available for identical species, and species with RAG-1 data are shown. There are 1742 characters (212 ribosomal [147 PI]; 1530 RAG-1 [624 PI]). Both methods produce identical trees, but parsimony produces an additional shortest tree in which hynobiids are monophyletic (length = 3061, CI = 0.4352, RI = 0.6589).

on the presence of shared, correlated larval traits in the adult morphology. Our results suggest that this is not the case. In our analyses, only three of the four paedomorphic families cluster together, and the level of support for this clade of paedomorphic species is weak (Figs. 2, 3).

Examination of character distributions among taxa suggests a possible explanation. Few of the putatively larval traits that occur in adults of paedomorphic species are shared among all paedomorphic species (e.g., external gills are absent in amphiumids and cryptobranchids) and paedomorphic species differ considerably in their proportion of larval traits (Duellman and Trueb, 1986; Rose, 1999; Wiens, unpublished). The cryptobranchids, for example, have adult cranial morphology similar to that of transforming salamanders (Duellman and Trueb, 1986; Rose, 1999), and cryptobranchids fail to cluster with other paedomorphic species in analyses of adult morphology (Figs. 2, 3). Thus, there seemingly is a continuum between the adult morphology of metamorphosing and nonmetamorphosing species (Rose, 1999), making the impacts of paedomorphosis on phylogenetic analysis more diverse, complicated, and difficult to discern.

Surprisingly, our results reveal two factors associated with paedomorphosis in salamanders that may have as much impact on phylogenetic analysis as the sharing of larval traits among adults of unrelated taxa. First, paedomorphic species not only share generalized larval traits, but also fail to develop clade-specific adult morphological traits that would allow their correct placement within a given group (e.g., diagnostic characters for a given family which are expressed only in post-metamorphic ontogeny). For example, placement of *Eurycea neotenes* with the paedomorphic families is only weakly supported in parsimony analysis of adult morphology (Fig. 2), but there is strong support for the monophyly of plethodontids excluding this species (bootstrap = 96%). Furthermore, parsimony trees based on morphological data show moderately strong support (bootstrap = 69%) for placing *E. longicauda* with *Pseudotriton montanus*. Both *Pseudotriton* and *Eurycea* belong to the tribe Hemidactyliini (Wake, 1966). Phylogenetic analysis of the molecular data (Fig. 4) shows strong support for monophyly of included Hemidactyliini and for placing *E. neotenes* with *E. longicauda*. Thus, the exclusion of *E. neotenes* from Plethodontidae and Hemidactyliini almost certainly is wrong and yet is statistically well supported by the morphological data. Similarly, Bayesian analysis of the morphological data shows strong support for monophyly of both plethodontids and hemidactyliines excluding *E. neotenes* (Fig. 3).

What causes this result? Parsimony and Bayesian analyses of the adult morphological data, excluding the 30 putatively larval (paedomorphic) characters, both produce trees in which *E. neotenes* is the sister group to all other plethodontids but is not placed with *E. longicauda*

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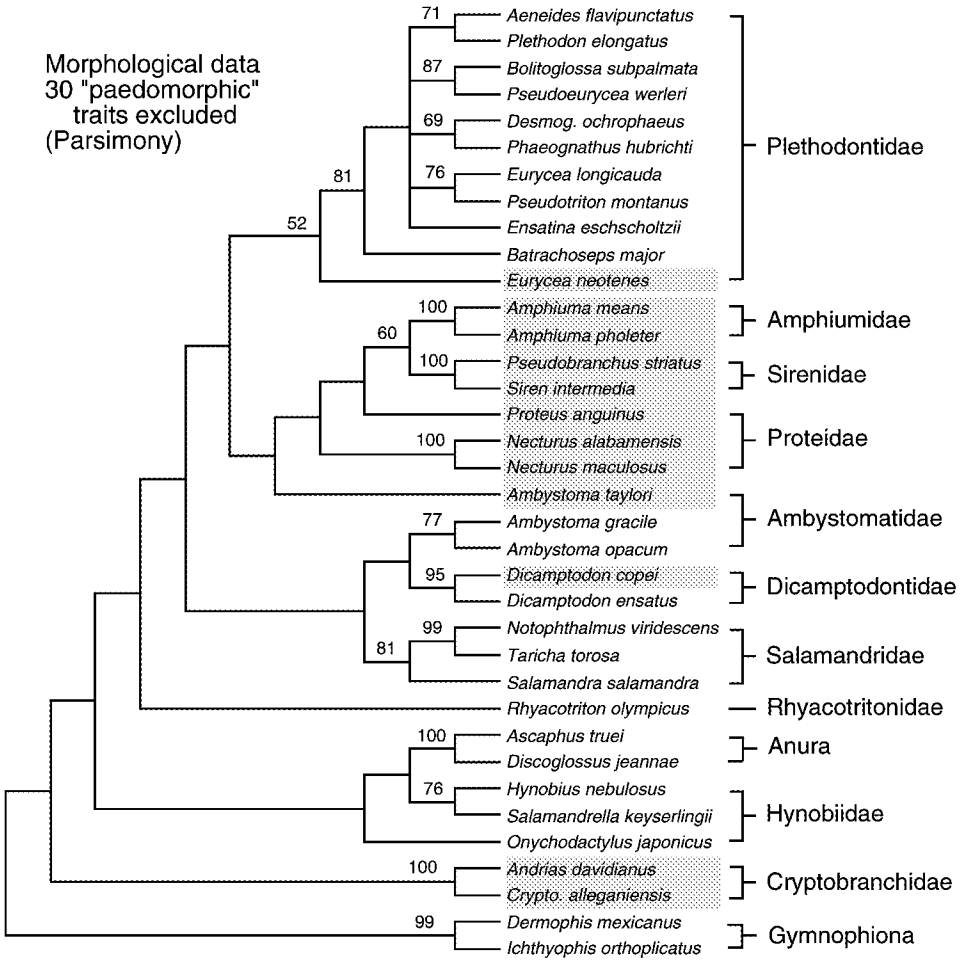


FIGURE 5. Salamander relationships based on parsimony analysis of adult morphological characters, with 30 putative paedomorphic characters excluded (paedomorphic taxa shaded). Note that most paedomorphic families are still placed in a single clade. Numbers above branches indicate bootstrap values $\geq$50%. There are 287 characters (268 PI) and three shortest trees (consensus tree shown) with length = 965.5, CI = 0.3353, and RI = 0.6113. Excluded paedomorphic characters include cranial osteology (n = 13; including absence of maxilla, septomaxilla, prefrontal, and nasal), hyoid morphology (n = 11; including presence of ceratobranchials II to IV), and external morphology (n = 6; including presence of gills, absence of eyelids).

(Figs. 5, 6). Thus, the repeated appearance of these larval traits in the adult stage of distantly related taxa cannot be the only factor responsible. Instead, individuals of *E. neotenes* fail to develop many of the diagnostic characters of plethodontids and hemidactyliines which appear only in adults, particularly characters of cranial, hyobranchial, and external morphology, such as presence of vomerine tooth patches on the parasphenoid (character 52), absence of the pterygoid (character 57), an elongate ceratobranchial I (character 174), and a nasolabial groove (character 278). These characters exemplify the difficulty in identifying and deleting suspected paedomorphic traits prior to a phylogenetic analysis; most salamander species lack these traits because they never evolved them, whereas *E. neotenes* lacks them because it is paedomorphic (i.e., these characters are misleading in paedomorphic plethodontids, but not in salamanders in general). Despite lacking these external, cranial, and hyobranchial synapomorphies, individuals of *E. neotenes* nevertheless retain plethodontid synapomorphies involving the limbs and vertebrae (e.g., characters 209, 222, 243, 263), regions of the body which seem to develop essentially adult morphology before metamorphosis (e.g., Wilder, 1925; Worthington and Wake, 1971; Duellman and Trueb, 1986). The case of *E. neotenes* strongly suggests that analysis of paedomorphic species can be problematic because of the absence of clade-specific adult traits that would allow their correct placement, rather than just the misleading presence of larval traits shared among all paedomorphic species. Therefore, the problem of paedomorphosis may be very difficult to detect and may not be solved by simply excluding putative paedomorphic characters. In a similar vein, previous authors (e.g., Kluge, 1989) have also suggested that paedomorphosis may increase the number of reversals; however, these reversals may not be detected as such if an analysis is misled by paedomorphosis in the first place (as in this study).

Finally, the results suggest an important but indirect effect of paedomorphosis on salamander morphology and phylogenetics. Somewhat surprisingly, when

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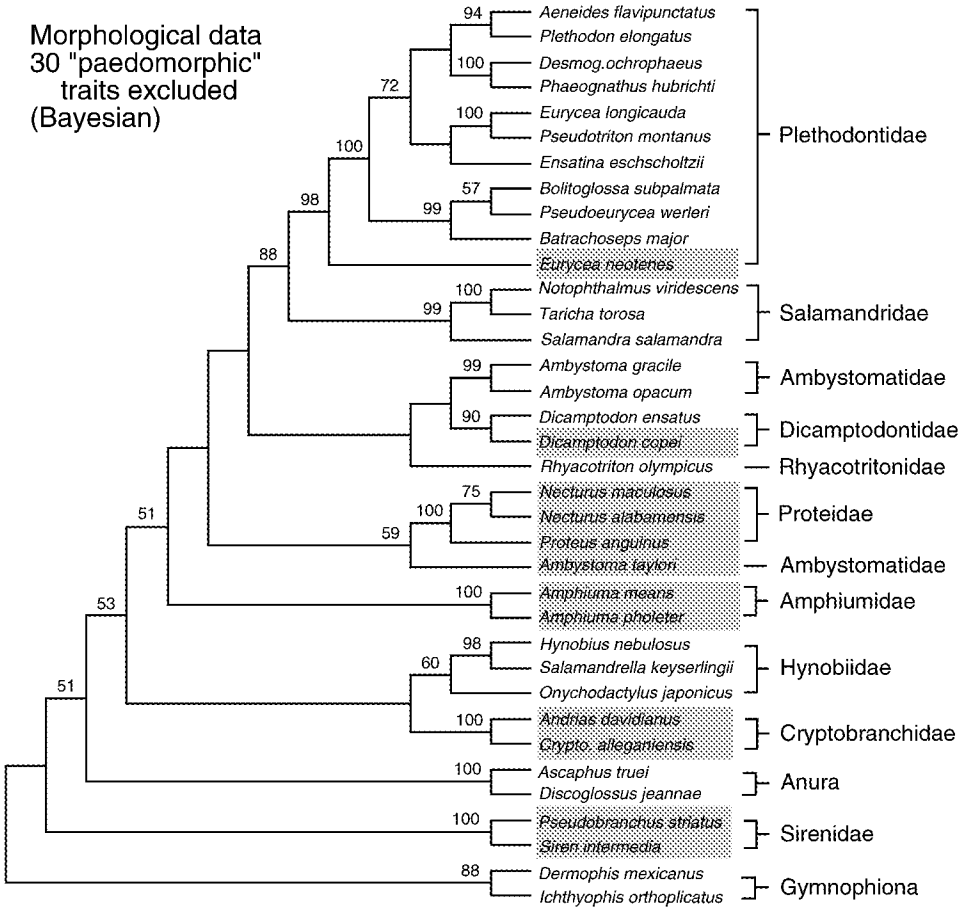


FIGURE 6. Salamander relationships based on Bayesian analysis of adult morphological characters, with 30 putative paedomorphic characters excluded (paedomorphic taxa shaded). Numbers above branches indicate posterior probability values $\geq$50%.

30 putatively paedomorphic traits are excluded from the parsimony analysis of adult morphology, the same three problematic paedomorphic families (Amphiumidae, Proteidae, Sirenidae) are still placed in a single clade (Fig. 5). Many of the remaining characters that support grouping these families in our analysis are associated with either increased ossification of the hyoid skeleton (characters 117, 130, 158, 166), modification of the vertebrae (characters 233, 235, 236, 246, 248, 254, 255), body elongation (characters 231, 232), or limb reduction (characters 208, 220). None of the four types of changes seem to be direct effects of paedomorphosis, and all four may be related to aquatic habitat use. For example, some of the characters of increased hyobranchial ossification and vertebral modification also appear in the aquatic, non-paedomorphic salamandrids (e.g., characters 117, 130, 158, 248). Furthermore, body elongation and limb reduction (see Fig. 1E, F) occur together in several distantly related groups of aquatic tetrapods (e.g., mosasaurs, cetaceans, sirenians), as well as burrowers, and these changes may facilitate aquatic locomotion (Carroll, 1988). Although many lineages of salamanders are aquatic as larvae, the extent to which larvae can adapt to aquatic habitats in nonpaedomorphic species may be limited by the morphological needs of the terrestrial adult stage. For example, the adult patterns of vertebral and digit numbers appear to develop and become fixed prior to metamorphosis (Wilder, 1925; Worthington and Wake, 1971; Duellman and Trueb, 1986). In contrast, paedomorphic salamander lineages lack the constraints imposed by terrestrial adult morphology and can more freely adapt to the aquatic niche. Thus, the potential for convergent aquatic adaptations in paedomorphic salamanders may represent an important indirect effect of paedomorphosis. The repeated and correlated loss of structures in different lineages associated with invasion of niches may be a general problem in morphological phylogenetics (e.g., limbless burrowing squamates; Lee, 1998).

Bayesian analysis of the reduced morphological data presents a somewhat different story. In one of the two Bayesian analyses (Fig. 6), the clade of Amphiumidae, Proteidae, and Sirenidae is broken up when the 30 "paedomorphic" characters are excluded. In the other replicated analysis (not shown), proteids and sirenids are still grouped together. The failure of the amphiumids, proteids, and sirenids to cluster may indicate that the Bayesian analysis is less sensitive than parsimony to the adaptive homoplasies associated with aquatic habitat; a potentially similar example of the robustness of this

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maximum likelihood model to adaptive convergence is shown by Lewis (2001). Nevertheless, many of the paedomorphic taxa clearly are misplaced in both analyses (e.g., *Ambystoma taylori, Eurycea neotenes*), and it appears that Bayesian analysis is strongly misled by the presence of shared larval traits (Fig. 3) and the absence of adult synapomorphies in paedomorphic taxa (e.g., placement of *Eurycea neotenes*; Fig. 5).

What Is the Phylogeny of Salamanders?

We present new parsimony and Bayesian analyses of salamander phylogeny based on combined molecular and morphological data (Figs. 7, 8). Our analyses incorporate the morphological and nuclear ribosomal data used by previous authors (e.g., Duellman and Trueb, 1986; Larson and Dimmick, 1993; Gao and Shubin, 2001) but also include a new molecular data set (1.5 kb of RAG-1), use of model-based methods for both molecular and morphological data, a greatly expanded set of morphological characters, and application of a conservative method for removing the misleading effects of paedomorphosis from the morphological data.

Our results are reassuringly similar to the combined-data tree of Larson and Dimmick (1993; their Fig. 3), and we provide strong support for many relationships that were only weakly supported in the bootstrap analyses of Larson and Dimmick (1993), including monophyly of Salamandroidea (caudates exclusive of sirenids, cryptobranchids, and hynobiids) and the clade Amphiumidae + Plethodontidae. We also conclusively resolve Rhyacotritonidae as the sister taxon of Amphiumidae + Plethodontidae (100% bootstrap and posterior probability values). The position of Rhyacotritonidae was incompletely resolved in the analysis of Larson and Dimmick (1993). Our results also provide conclusive phylogenetic support for the monophyly of Proteidae (*Necturus* and *Proteus*), which was controversial in the earlier literature (e.g., Larsen and Guthrie, 1974; Hecht and Edwards, 1976; see also Trontelj and Goricki, 2003).

Despite many similarities, our results also show two interesting differences from those of Larson and Dimmick (1993). First, we show the possibility that Cryptobranchoidea (Cryptobranchidae + Hynobiidae) is the sister taxon of all other salamanders, rather than Sirenidae. This result is equivocal in the parsimony analysis but strongly supported in the Bayesian analysis. Some simulation studies suggest that Bayesian analyses may sometimes accord unduly high support values to questionable or incorrect branches (e.g., Alfaro et al., 2003; Cummings et al., 2003). However, Bayesian analysis may often provide more accurate estimates of phylogeny than parsimony because it incorporates explicit

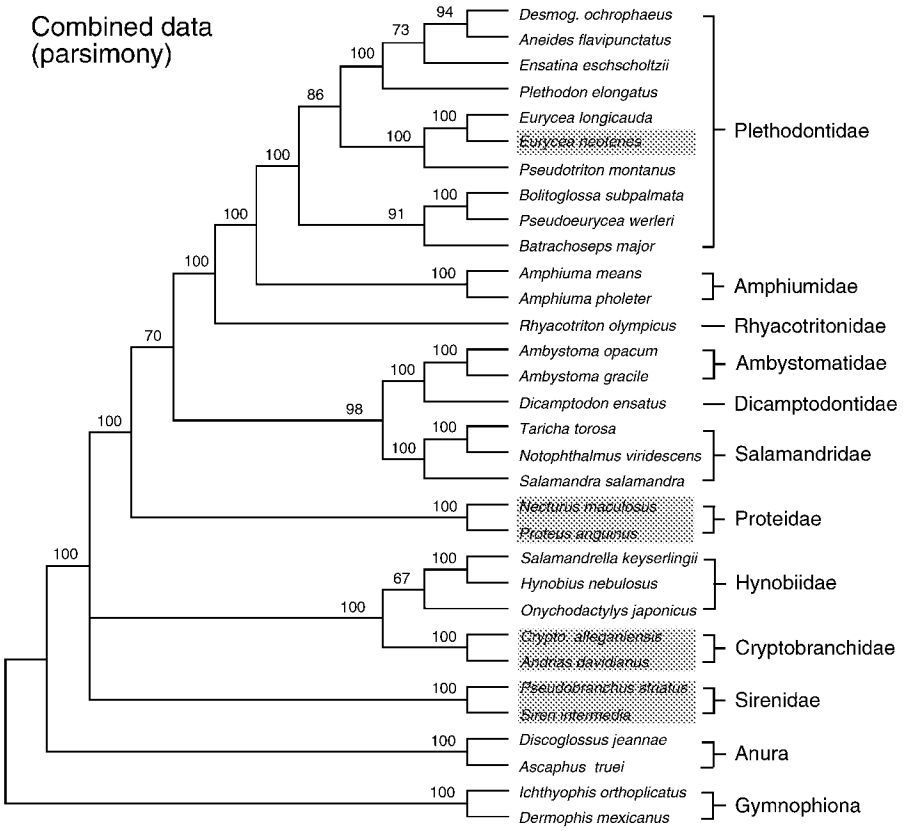


FIGURE 7. Salamander relationships based on parsimony analysis of the combined molecular and morphological data, with paedomorphic taxa (shaded) coded as unknown for adult morphology. There are 2068 characters (992 PI; 326 morphological [221 PI]; 212 ribosomal [147 PI]; 1530 RAG-1 [624 PI]) and 2 shortest trees (consensus tree shown) with length = 3763.0, CI = 0.4323, and RI = 0.6552. Numbers above branches indicate bootstrap values ≥50%. For some taxa, identical species were not available for all three data sets, and only species coded for morphology are shown.

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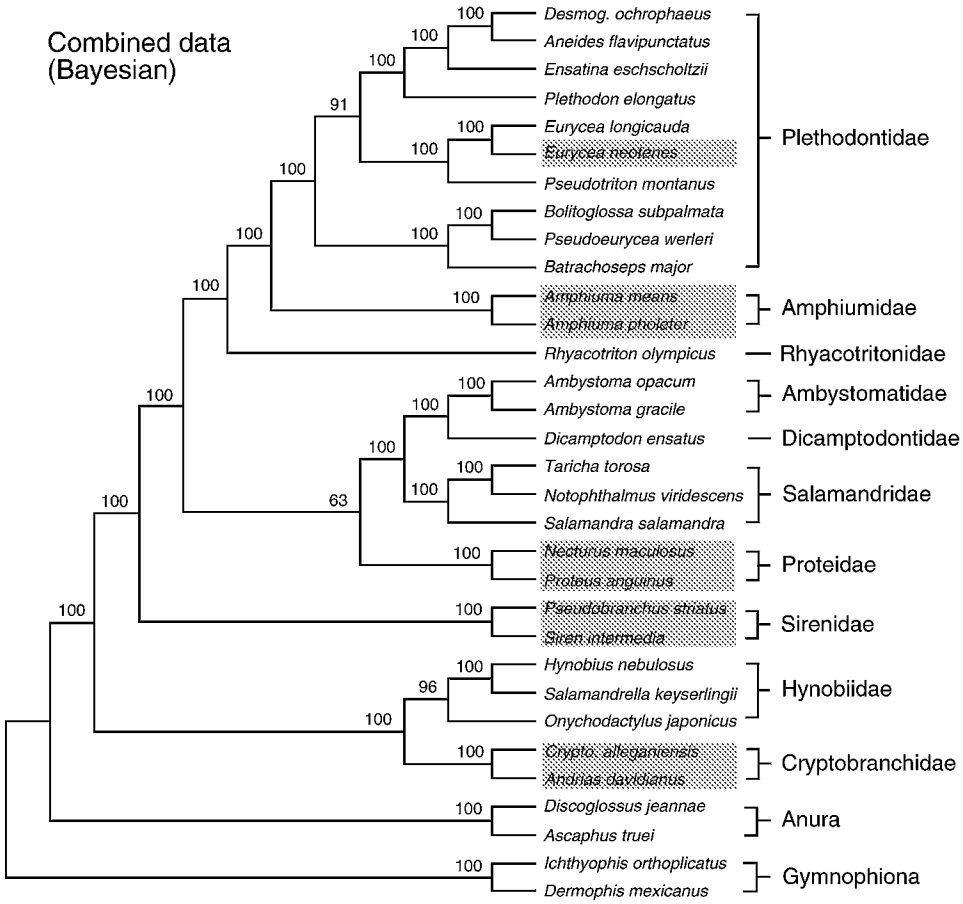


FIGURE 8. Salamander relationships based on Bayesian analysis of the combined molecular and morphological data, with paedomorphic taxa (shaded) coded as unknown for adult morphology. There are 2068 characters (992 PI; 326 morphological [221 PI]; 212 ribosomal [147 PI]; 1530 RAG-1 [624 PI]). Numbers above branches indicate posterior probabilities $\geq$50%. For some taxa, identical species were not available for all three data sets, and only species coded for morphology are shown.

models of DNA sequence evolution and may be less sensitive to long-branch attraction (e.g., Alfaro et al., 2003).

Our results also suggest some ambiguity concerning the placement of Proteidae. Our Bayesian analysis shows some support for placing Proteidae as sister taxon of (Salamandridae + (Dicamptodontidae + Ambystomatidae)), as did the parsimony analysis of Larson and Dimmick (1993). However, our parsimony analysis shows support for a more basal placement of proteids. Although salamander relationships now seem relatively well understood at the family level (e.g., relative to many other animal clades), additional data would be useful to clarify the placement of sirenids and proteids. We tentatively favor the results of the Bayesian analysis (Fig. 8) as our preferred hypothesis of salamander relationships, given the potential advantages of model-based methods relative to parsimony.

CONCLUSIONS

In summary, our results demonstrate the dangers of ignoring developmental processes when reconstructing phylogenies based on morphological data. Major developmental changes, such as paedomorphosis in salamanders, may be an important and underappreciated source of error in morphological phylogenetics in many groups of organisms. Paedomorphosis, for example, is thought to be a widespread mechanism of phenotypic change in groups ranging from plants to primates (Gould, 1977; McNamara, 1996). Our results show that the effects of paedomorphosis on phylogenetic analysis can be complex and can produce results that apparently are both incorrect and statistically well supported (e.g., monophyly of plethodontids excluding *Eurycea neotenes*). These effects may be difficult to detect and to correct analytically and are unlikely to be solved by simply excluding suspected problematic characters (Figs. 5, 6). Our results also demonstrate that likelihood-based methods for morphological data may be just as easily misled by this problem as is parsimony. The impact of large-scale developmental changes may be most apparent in cases of incongruence between trees from molecular and morphological data and may explain the disparate results obtained from these data sets in some cases. This issue should be of special concern for analyses of fossil taxa, for which estimates of phylogeny independent of morphology may

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be unavailable. Our study also provides an improved estimate of salamander phylogeny for use in all disciplines of biology that utilize salamanders as model systems.

ACKNOWLEDGMENTS

We thank A. Larson and W. Dimmick for providing an electronic version of their data set, and the following curators and collection managers for loan of specimens and permission to make skeletal preparations (institutional abbreviation): C. J. Cole, L. Ford (AMNH), J. Vindum (CAS), S. Rogers (CM), M. Kearney, A. Resetar, and H. Voris (FMNH), B. Hollingsworth (SDNHM), D. Wake (MVZ), S. Busack (NCSM), D. Cannatella (TNHC), H. Dundee (TU), D. Auth (UF), R. Nussbaum, G. Schneider (UMMZ), and J. Campbell (UTA). For use of tissues we thank J. Bernardo, R. Highton, T. LaDuke, W. Leonard, T. Reeder, B. Sket, E. Smith, S. Trauth, P. Trontelj, W. Van Devender, D. Weisrock, and MVZ. We are grateful to J. Dermid, T. Leenders, B. Moon, and W. Van Devender for allowing us to use their salamander photos. For many helpful comments on the manuscript we thank F. A. Anderson, L. Buckley, A. Collins, R. Geeta, R. Page, D. Wake, and members of the Wiens lab. We thank the U.S. National Science Foundation for financial support (DEB 0331747 to J.J.W. and 0129242 to P.T.C.).

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First submitted 4 February 2004; reviews returned 2 April 2004; final acceptance 8 June 2004
Associate Editor: Frank (Andy) Anderson

APPENDIX 1

Specimens examined for morphological analyses. Institutional abbreviations follow Leviton et al. (1985).

Osteological (cleared-and-stained unless noted)

Ingroup (Caudata): Ambystomatidae: *Ambystoma gracile:* CM 38830. *Ambystoma opacum:* CM 113804. *Ambystoma taylori:* MVZ 184847. Amphiumidae: *Amphiuma means:* CM 113668. *Amphiuma pholeter:* UF 28813. Cryptobranchidae: *Andrias davidianus:* SDNHM 55583 (dry skeletal), 67243. *Cryptobranchus alleganiensis:* CM 4054, 92272 (dry skeletal). Dicamptodontidae: *Dicamptodon copei:* MVZ 192677. *Dicamptodon ensatus:* CM 51566, MVZ 22517 (dry skeletal). Hynobiidae: *Hynobius nebulosus:* CAS 26161. *Onychodactylus japonicus:* CM 68237, UMMZ 183395. *Salamandrella keyserlingii:* MVZ 222334. Plethodontidae: *Aneides flavipunctatus:* CM 55779. *Batrachoseps major:* CM 135016. *Bolitoglossa subpalmata:* CM 62414. *Desmognathus ochrophaeus:* CM 46553GG, 46553AY. *Ensatina eschscholtzii:* CM 55777, 26002. *Eurycea neotenes:* TNHC 52766-67. *Eurycea longicauda:* CM 113842. *Phaeognathus hubrichti:* CM 135682. *Plethodon elongatus:* CM 33091. *Pseudoeurycea werleri:* CM 135697. *Pseudotriton montanus:* CM 37257. Proteidae: *Necturus alabamensis:* UF 72110. *Necturus maculosus:* CM 137004, 137006. *Proteus anguinus:* AMNH 13482, MVZ 129392 (dry skeletal), 184990 (dry skeletal). Rhyacotritonidae: *Rhyacotriton olympicus:* MVZ 173355, 173357. Salamandridae: *Notophthalmus viridescens:* CM 126955, 139068. *Salamandra salamandra:* CM 60797R. *Taricha torosa:* CM 55783. Sirenidae: *Pseudobranchus striatus:* CM 20130V. *Siren intermedia:* CM 49281.

Outgroups: Anura: Ascaphidae: *Ascaphus truei:* CM 39867. Discoglossidae *Discoglossus jeannae:* CM 54680. Gymnophiona: Caeciliidae: *Dermophis mexicanus* CM 58194 (dry skeletal). Ichthyophiidae: *Ichthyophis orthoplicatus:* CM 93638.

External

Ingroup (Caudata): Ambystomatidae: *Ambystoma gracile:* CM 7152, 58172, 62254. *Ambystoma opacum:* CM 19472E, 19473C. *Ambystoma taylori:* CM 39981. Amphiumidae: *Amphiuma means:* CM 18812, 135670. *Amphiuma pholeter:* UF 28810-11. Cryptobranchidae: *Andrias davidianus:* CM 56449. *Cryptobranchus alleganiensis:* CM 3747, 21651. Dicamptodontidae: *Dicamptodon copei:* CM 3781. *Dicamptodon ensatus:* CM 62286. Hynobiidae: *Hynobius nebulosus:* CAS 26169, 26254, 26298, 26326. *Onychodactylus japonicus:* CM 60644, 68238. *Salamandrella keyserlingii:* CM 47583-84. Plethodontidae: *Aneides flavipunctatus:* CM 51578, 51580. *Batrachoseps major:* CM 62370, 62373. *Bolitoglossa subpalmata:* CM 62424, 62427. *Desmognathus ochrophaeus:* CM 5873K, 74489, 74589. *Ensatina eschscholtzii:* CM 25972, 25985, 25998. *Eurycea longicauda:* CM 113851, 113854. *Eurycea neotenes:* MVZ 119967, 120081. *Phaeognathus hubrichti:* CM 135684-85. *Plethodon elongatus:* CM 7155-56. *Pseudoeurycea werleri:* CM 135699, 135701. *Pseudotriton montanus:* CM 139402, 139404. Proteidae: *Necturus alabamensis:* UF 69263, 72111-12. *Necturus maculosus:* CM 30110, 75895-96. *Proteus anguinus:* CM 21948. Rhyacotritonidae:

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Rhyacotriton olympicus: MVZ 185912, 185914. Salamandridae: *Notophthalmus viridescens:* CM 46536CA, 46536 BL. *Salamandra salamandra:* CM 52132, 54276. *Taricha torosa:* CM 51572, 135050. Sirenidae: *Pseudobranchus striatus:* CM 12175, 20131B. *Siren intermedia:* CM 49284.

Outgroups: Anura: Ascaphidae: *Ascaphus truei:* CM 30686, 39868. Discoglossidae: *Discoglossus jeannae:* CM 53884 C. Gymnophiona: Caeciliidae: *Dermophis mexicanus:* CM 90093. Ichthyophidae: *Ichthyophis orthoplicatus:* CM 93646.

APPENDIX 2

Nonmolecular characters (morphological, life history, chromosomal) used in phylogenetic analyses. Citations emphasize the first usage of a character in an explicit phylogenetic analysis. Anatomical terminology generally follows Duellman and Trueb (1986).

Skull

1. Premaxillae: (0) not fused at base, (1) fused at base. Modified from character A of Duellman and Trueb (1986).
2. Pars dentalis of premaxilla: (0) present lateral to pars dorsalis, (1) absent lateral to pars dorsalis.
3. Premaxilla: (0) dentate, (1) edentate. Character EE of Duellman and Trueb (1986).
4. Pars dorsalis of premaxilla: (0) not contacting frontals, (1) contacting frontals.
5. Pars dorsalis of premaxilla: (0) not elongate, (1) elongate, separating vomers ventrally.
6. Pars dorsalis of premaxilla: (0) not contacting medially, (1) contacting medially.
7. Pars dorsalis of premaxilla: (0) separated to expose fontanelle, (1) contacting medially throughout their lengths.
8. Pars dorsalis of premaxilla: (0) not fused, (1) fused partly of fully. Modified from character A of Duellman and Trueb (1986).
9. Pars dorsalis of premaxilla, combined width: (0) less than interorbital width, (1) greater than interorbital width.
10. Pars dorsalis of premaxilla: (0) not overlapping medially, (1) overlapping medially.
11. Bony lamina between pars dorsalis of premaxilla: (0) absent, (1) present.
12. Pars palatina of premaxilla: (0) absent, (1) present.
13. Premaxilla-vomer contact: (0) absent, (1) present (usually involving pars palatina of premaxilla).
14. Maxilla: (0) present, (1) absent. Character C of Duellman and Trueb (1986).
15. Maxilla: (0) dentate, (1) reduced, edentate. Character C of Duellman and Trueb (1986).
16. Posterior process of maxilla: (0) dentate, (1) edentate.
17. Process from pars dentalis of maxilla overlaps premaxilla: (0) no, (1) yes.
18. Anterior margin of pars facialis of maxilla: (0) posterior to external naris, (1) covers posterior portion of external naris.
19. Maxillary arcade: (0) complete, continuous row of elements from premaxilla to jaw articulation, (1) incomplete. From Trueb and Cloutier (1991).
20. Septomaxilla: (0) present, (1) absent. Character D of Duellman and Trueb (1986).
21. Septomaxilla: (0) inside of external naris (posterior end covered by alary cartilage), (1) outside of external naris (posterior end dorsal to alary cartilage).
22. Posterior end of septomaxilla: (0) not contacting other cranial elements, (1) contacting maxilla, (2) contacting prefrontal, (3) nasal. Unordered. In the *Ambystoma maculatum* examined, the septomaxilla contacts both the maxilla and prefrontal, and was coded as having both states ("1,2").
23. Prefrontal: (0) present, (1) absent. Character 11 of Gao and Shubin (2001).
24. Prefrontal: (0) does not contacts parietal, (1) contacts parietal.
25. Prefrontal, posterior processes project into orbit: (0) absent, (1) present
26. Prefrontal: (0) lies on top of nasal capsule only, (1) extends posteroventrally to form posterior border of nasal capsule.
27. Lacrimal: (0) present, (1) absent. Character F from Duellman and Trueb (1986).
28. Nasal: (0) present, (1) absent. Modified from character E of Duellman and Trueb (1986).
29. Medial articulation of nasals: (0) present, (1) absent. Modified from character B of Duellman and Trueb (1986).
30. Nasal-prefrontal contact: (0) present, (1) absent. Character 60 of Gao and Shubin (2001).
31. Nasal and maxilla: (0) contacting or abuttting, (1) separated.
32. Nasals, relationship to pars dorsalis of premaxilla: (0) overlain or not in contact, (1) nasals overlie pars dorsalis, at least in part.
33. Nasal: (0) squarish, not elongate, (1) slender and elongate.
34. Nasal: (0) separate from frontal, (1) partly or completely fused to frontal.
35. Nasal: (0) not forked posteriorly, (1) forked posteriorly, with dorsal and ventral processes enclosing orbitonasal foramen.
36. Frontal: (0) does not contact maxilla, (1) contacts maxilla. Character 33 of Gao and Shubin (2001).
37. Frontal, dermostosis: (0) absent, (1) present. Modified from character 30 of Gao and Shubin (2001).
38. Frontoparietal fontanelle: (0) absent, (1) present.
39. Dorsolateral shelf on frontal: (0) absent, (1) present.
40. Posterior edge of parietals, extends between exoccipitals to edge of foramen magnum on tectum synocticum: (0) no, (1) yes.
41. Ventrolateral extension of parietal covers orbitosphenoid region anteriorly (in lateral view): (0) absent, (1) present. Modified from character 34 of Gao and Shubin (2001).
42. Parietal and exoccipital: (0) not forming casque around foramen magnum, (1) forming casque around foramen magnum.
43. Vomer: (0) with postchoanal process, (1) without postchoanal process.
44. Vomer: (0) with prechoanal process, (1) without prechoanal process.
45. Vomer: (0) not articulating with pterygoid, (1) articulates with pterygoid.
46. Vomers: (0) separated anteriorly and medially, exposing fontanelle, (1) in contact anteromedially, no fontanelle exposed.
47. Vomer, posterior dorsal process extending onto orbitosphenoid: (0) absent, (1) present.
48. Placement of vomerine teeth: (0) medial, (1) marginal (adjacent and parallel to max. and premaxillary teeth), (2) teeth centrally located on vomer. Modified from character S of Duellman and Trueb (1986). Unordered.
49. Vomerine teeth: (0) arranged in single row, (1) arranged in multiple rows anteriorly (on vomer), (2) arranged in single rows anteriorly, in multiple rows on parasphenoid. Unordered.
50. Vomerine teeth: (0) oriented perpendicular to body axis or curved, (1) parallel to body axis.
51. Vomerine tooth series: (0) extends posteriorly onto parasphenoid, (1) no posterior extension. Modified from character S of Duellman and Trueb (1986). Species with marginal vomerine teeth were coded as unknown for this character.
52. Vomerine teeth: (0) not extending posteriorly length of orbit, (1) extending posteriorly, teeth on parasphenoid continuous with teeth on vomer, (2) teeth on vomer separate from tooth patches on parasphenoid.
53. Vomerine teeth: (0) present on postchoanal process, (1) absent on postchoanal process. Character from Tihen (1958).
54. Vomerine teeth: (0) continuous row, (1) separate patch of teeth on postchoanal ramus.
55. Small bony element dorsal to postchoanal ramus of vomer: (0) absent (including taxa with no postchoanal ramus), (1) present.
56. Palatine: (0) absent, (1) present. In caudates, this is a small dentate element just posterior to the vomers that is fused with the pterygoid in some taxa.
57. Pterygoid: (0) present, (1) absent. Modified from character I of Duellman and Trueb (1986).
58. Palatine and pterygoid: (0) separate (or only one element present), (1) elements present, articulating or adjacent but not fused, (2) fused (as indicated by presence of single element, dentate anteriorly, extending posteriorly to level of jaw articulation).

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59. Pterygoid, anteromedial process that articulates with parasphenoid: (0) absent, (1) present. Modified from character 9 of Gao and Shubin (2001).
60. Pterygoid, with distinct anterolateral process: (0) present, roughly triradiate, (1) absent, roughly rectangular. Modified from character 9 of Gao and Shubin (2001).
61. Pterygoid (0) not greatly reduced, (1) reduced to tiny element. Modified from character I of Duellman and Trueb (1986). Unordered.
62. Pterygoid, anterior ramus: (0) free, not contacting maxilla, (1) not free, contacting or nearly contacting maxilla.
63. Anterior margin of pterygoid: (0) smooth, (1) serrate, with irregular projections.
64. Posteriorly directed process on anterior ramus of pterygoid: (0) absent, (1) present.
65. Pterygoid and coronoid process of prearticular: (0) well-separated, (1) articulating or nearly contacting.
66. Posterior margin of pterygoid extends posterior to jaw articulation: (0) no, (1) yes.
67. Pterygoid, with dorsomedial process that articulates with orbitosphenoid and forms foramen posterior to optic foramen: (0 absent, (1) present.
68. Squamosal: (0) does not contact frontal, (1) contacts frontal. Character GG from Duellman and Trueb (1986).
69. Squamosal-parietal: (0) separated or barely contacting, (1) in contact. Modified from character 37 of Gao and Shubin (2001).
70. Squamosal: (0) not expanded ventrally, (1) expanded ventrally, occupies articular region.
71. Squamosal, main shaft (lateral view): (0) oriented roughly vertically, (1) oriented diagonally, with dorsoposterior inclination.
72. Hook-like (ventrally-directed) process on dorsal head of squamosal: (0) absent, (1) present.
73. Columellar process of squamosal (connecting stapes and squamosal): (0) absent, (1) present. Character from Larsen and Guthrie (1974).
74. Quadrate ossification: (0) present, (1) absent.
75. Posterior process on pars quadrati of quadrate: (0) absent, (1) present.
76. Jaw articulation: (0) well ventral to level of ventral margin of braincase, (1) at level of ventral margin of braincase.
77. Atlanto-mandibular ligament: (0) absent, (1) present. Character from Wake (1966).
78. Quadrate-parasphenoid articulation: (0) absent, (1) present. Character from Wake (1966).
79. Parasphenoid: (0) not extending laterally beyond level of orbitosphenoid, (1) extending laterally beyond level of orbitosphenoid.
80. Orbitosphenoid: (0) present, (1) absent.
81. Optic foramen: (0) enclosed in bone anteriorly or not at all, (1) enclosed entirely in bone.
82. Orbitosphenoid: (0) not extending lateral to frontals, or extending only slightly anteriorly, (1) extending well lateral to frontals throughout their length.
83. Lateral crests on posterior of skull: (0) absent, (1) present.
84. Mid-saggital crest on posterior of skull: (0) absent, (1) present.
85. Exoccipitals: (0) separated medially at tectum synocticum, (1) fused.
86. Exoccipitals: (0) closely approaching each other on tectum synocticum, (1) exoccipitals barely extending to tectum synocticum, widely separated.
87. Posteriormost margin of auditory capsules: (0) anterior to occipital condyles, (1) posterior to occipital condyles.
88. Fusion of opisthotic and exoccipital: (0) absent, (1) present. Modified from character H of Duellman and Trueb (1986).
89. Lateral flange on prootic (extending to squamosal): (0) absent, (1) present.
90. Fusion of opisthotic and prootic: (0) absent, (1) present (partial to complete). Modified from character H of Duellman and Trueb (1986).
91. Occipital condyles: (0) not stalked, (1) stalked.
92. Operculum: (0) present, (1) absent. Modified from character K of Duellman and Trueb (1986).
93. Stapes: (0) present, (1) absent. Modified from character K of Duellman and Trueb (1986).

Cranial Cartilages

94. Alary cartilage: (0) present, (1) absent.
95. Antorbital cartilage: (0) present, (1) reduced or absent.
96. Large foramen at lateral end of antorbital cartilage: (0) absent, (1) present.
97. Small foramina in antorbital cartilage: (0) absent, (1) present.
98. Foramen in oblique cartilage, posterior to narial fenestra: (0) absent, (1) present.
99. Acuminate, anterior process from pars quadrati: (0) absent, (1) present.
100. Pterygoid cartilage: (0) present, (1) absent.
101. Pterygoid cartilage: (0) reaches to maxilla, (1) does not reach to maxilla. Character from Kraus (1988).
102. Pterygoid cartilage, dorsal process anteriorly: (0) absent, (1) present.
103. Pterygoid cartilage: (0) rounded in cross section, (1) flattened and expanded.
104. Anterior median process on internasal tectum: (0) absent, (1) present.

Lower Jaw

105. Angular: (0) present, (1) absent (presumably fused to prearticular). Character O of Duellman and Trueb (1986).
106. Coronoid: (0) absent, (1) present.
107. Coronoid: (0) dentate, (1) edentate.
108. Articular: (0) present, (1) absent (unossified).
109. Meckel's cartilage: (0) does not extend to mandibular symphysis, (1) extends to mandibular symphisis.
110. Mandible: (0) thickened at symphysis, (1) thinner at symphysis (in anterior view).
111. Retroarticular process: (0) absent, (1) present.
112. Coronoid process of prearticular: (0) present, (1) absent.
113. Coronoid process of prearticular: (0) adjacent to jaw articulation, (1) distinctly anterior to jaw articulation.
114. Coronoid process of dentary: (0) absent, (1) present.
115. Dentary: (0) dentate, (1) edentate.
116. Dentary teeth: (0) pedicellate, (1) non-pedicellate. Character T of Duellman and Trueb (1986).

Hyobranchial Skeleton

117. Ceratohyal: (0) cartilaginous, (1) ossified posteriorly only, (2) ossified throughout length. Ordered.
118. Ceratohyal: (0) anterior end not attached to other hyobranchial elements, (1) attached to other hyobranchial elements.
119. Ceratohyal: (0) expanded anteriorly, wider than other hyobranchial elements, (1) not expanded anteriorly, similar in width to other hyobranchial elements.
120. Ceratohyal, ventral process near posterior end: (0) absent, (1) present.
121. Anterior regions of ceratohyals: (0) dorsal to hyobranchial I and/or other hyobranchial elements, (1) lateral, not dorsal.
122. Ceratohyal, elongate anterolateral processes: (0) absent, (1) present.
123. Ceratohyal, elongate anterolateral processes: (0) separate medially, (1) overlapping medially.
124. Ceratohyal, elongate anterolateral processes: (0) not continuous posteriorly, (1) continuous posteriorly.
125. Ceratohyal, pointed anteromedial process: (0) absent, (1) present.
126. Ceratohyals: (0) not overlapping medially, (1) overlapping medially.
127. Posterior end of ceratohyal: (0) free, (1) attached (via ligament) to pars quadrati, (2) attached to posterolateral corner of skull roof. Unordered.
128. Posterior end of ceratohyal: (0) not recurved (oriented posteriorly or dorsally), (1) recurved (oriented anteriorly).
129. Cartilaginous element connecting (via ligament) ceratohyal and pars quadrati: (0) absent, (1) present.

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130. Basibranchial I: (0) cartilaginous, (1) ossified.
131. Basibranchial I, lateral process at anterior end: (0) absent, (1) present (T-shaped) but short, (2) present, lateral processes elongate. Ordered.
132. Anterior end of basibranchial I: (0) not forked, (1) forked, incised anteriorly (divided).
133. Ventral process on basibranchial I: (0) absent, (1) present.
134. Basibranchial I: (0) not expanded at mid-length, (1) expanded.
135. Lingual cartilage: (0) anterior extension of basibranchial I (extending past radius) and lingual cartilages absent, (1) anterior extension of basibranchial I present, lingual cartilage absent, (2) lingual cartilage present. Unordered.
136. Anterior end of basibranchial I: (0) not pinched and bulbous, (1) pinched and bulbous.
137. Anterior radius: (0) absent, (1) present.
138. Posterior radius: (0) absent, (1) present.
139. Anterior radius, anterior end: (0) free, (1) connected to ceratohyal.
140. Anterior end of anterior radius: (0) posterior to anterior tip of ceratohyal, (1) anterior to anterior tip of ceratohyal.
141. Anterior radius: (0) separate from basibranchial I, (1) continuous with basibranchial I.
142. Anterior radius: (0) projects anteriorly, (1) laterally, (2) projects posteriorly.
143. Anterior radius: (0) not expanded at base, (1) expanded at base.
144. Anterior radius: (0) not expanded distally, (1) expanded distally.
145. Anterior radii: (0) separate, (1) cartilaginous process links right and left anterior radii dorsally.
146. Corpus arcuata (otoglossal): (0) absent, (1) present, small, ring-shaped, (2) large, shield-shaped.
147. Basibranchial II: (0) present, (1) absent.
148. Basibranchial II: (0) connected to other hyobranchial elements anteriorly (generally basibranchial I), (1) free.
149. Basibranchial II; (0) well posterior to posterior edge of basibranchial I, (1) adjacent to posterior edge of basibranchial I. Taxa in which basibranchial I and II are connected are coded as unknown.
150. Basibranchial II, lateral processes: (0) absent, (1) present.
151. Basibranchial II, anterior process: (0) absent, (1) present.
152. Basibranchial II, process posterior to lateral processes: (0) absent, (1) present.
153. Posterior median process of basibranchial II: (0) short (including absent), (1) elongate, expanded in width posteriorly.
154. Lateral processes of basibranchial II: (0) tips unossfied, (1) tips ossified
155. Basibranchial II, lateral processes: (0) curved posterolaterally, (1) not curved, more-or-less perpendicular to body axis, (2) curved anterolaterally. Ordered.
156. Basibranchial II, maximum width (i.e., span of lateral processes): (0) much wider than width of basibranchial I, (1) greatly reduced in width, approximately equal to basibranchial I or narrower.
157. Basibranchial II: (0) single or no posterior processes, (1) two or more, irregular.
158. Hyobranchial I: (0) cartilaginous, (1) ossified.
159. Hyobranchials I: (0) separated medially, (1) in contact medially.
160. Hypobranchial I and ceratobranchial I: (0) separate, (1) fused into single rod. Character P of Duellman and Trueb (1986).
161. Groove and pocket on medial surface of hyobranchial I–ceratobranchial I: (0) absent, (1) present.
162. Anterior process on anterior end of hyobranchial I: (0) absent, (1) present.
163. Hyobranchials I and II: (0) separated, (1) in contact at basibranchial I.
164. Hyobranchial I: (0) thicker than hyobranchial II, (1) reduced in width, thinner than hyobranchial II.
165. Hyobranchial II: (0) present, (1) absent
166. Hyobranchial II: (0) cartilaginous, (1) ossified
167. Hyobranchial II: (0) not connected to ceratobranchial I, (1) connected to ceratrobranchial I.
168. Hyobranchial II: (0) not continuous with ceratobranchial I, (1) continuous with ceratrabranchial I.
169. Anterior end of hyobranchial II: (0) connected to basibrancial (I or II), (1) free.
170. Hyobranchial II: (0) rod-like, (1) reduced to small, spherical element.
171. Hypobranchial II, connected to ceratobranchial I: (0) medial surface, (1) anterior surface (adjacent to hyobranchial I).
172. Ceratobranchial I: (0) cartilaginous, (1) ossified.
173. Posterior end ceratobranchial I: (0) straight, (1) recurved
174. Ceratobranchial I: (0) not extending to suprascapula, (1) extends or nearly extends to suprascapula.
175. Posterior end of of ceratobranchial I, relationship to ceratobranchial II, (0) anterior, (1) ventral.
176. Ceratobranchial I, ventral process near posterior end: (0) absent, (1) present.
177. Ceratobranchial II: (0) present, (1) absent. Character Q of Duellman and Trueb (1986).
178. Ceratobranchial II: (0) cartilaginous, (1) ossified.
179. Ceratobranchial III: (0) present, (1) absent.
180. Ceratobranchial III: (0) cartilaginous, (1) ossified.
181. Ceratobranchial IV: (0) present, (1) absent.
182. Basihyal: (0) present, (1) absent.
183. Basihyals: (0) separated medially, (1) in contact medially.
184. Anterior hyoid element (basihyal): (0) paired, (1) divided into three or more elements.
185. Hypohyal: (0) present, (1) absent.
186. Cartilaginous element lateral to junction of ceratohyal and hypohyal: (0) absent, (1) present

Pectoral Girdle

187. Coracoid ossification: (0) present, (1) absent.
188. Scapula and coracoid ossification: (0) separate, (1) continuous. Modified from character U Duellman and Trueb (1986).
189. Ossification of coracoid, extending to anterior margin of pectoral girdle between coracoid and coracoid: (0) no, (1) yes.
190. Coracoids: (0) contacting medially, (1) separated medially.
191. Coracoids: (0) overlapping or not contacting, (1) fused medially.
192. Sternum: (0) present, (1) absent.
193. Procoracoid cartilage: (0) not elongate, with well-developed anteromedial process, (1) elongate with anteromedial process reduced.
194. Procoracoid and coracoid: (0) not overlapping anteriorly, (1) overlapping anteriorly, enclosing foramen.
195. Supracoracoid foramen: (0) present, (1) absent.
196. Supracoracoid foramen: (0) entirely in cartilage: (1) partly in bone, (2) entirely in bone. Ordered.
197. Suprascapula: (0) expanded in width dorsally, (1) not expanded, about same width as dorsal width of scapula.

Pelvic Girdle

198. Pelvic girdle: (0) present, (1) absent. Modified from character KK of Duellman and Trueb (1986).
199. Pelvic girdle: (0) halves fused medially, (1) halves separate medially.
200. Ypsiloid cartilage: (0) present, (1) absent. Character Y of Duellman and Trueb (1986).
201. Ypsiloid cartilage, anterolateral process: (0) roughly as long or longer than posterior median process, (1) distinctly shorter than median process, relatively flat and anteriorly oriented.
202. Triangular, cartilaginous extension of pubis: (0) absent, (1) present.
203. Lateral processes of pubis: (0) present, (1) absent.
204. Ossification of ischium: (0) not extending to anterior margin of pelvic girdle, (1) extending to anterior margin of pelvic girdle.
205. Ossification of ischia: (0) meeting mid-ventrally (separated by thin strip of cartilage), (1) well-separated mid-ventrally.
206. Posterior median process on ischium: (0) absent, (1) present.
207. Median processes of pubis: (0) posterior to or level with lateral processes, (1) anterior to lateral processes.

Forelimb

208. Number of fingers on forelimb: (0) four, (1) three, (2) two, (3) one. Ordered.
209. Number of phalanges on digit I of manus: (0) two, (1) one.
210. Crista dorsalis of humerus: (0) present, (1) absent.

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211. Carpals: (0) all elements cartilaginous, (1) some (but not all) ossified, (2) all elements at least partly ossified.
212. Carpals 1 and 2: (0) separate, (1) fused. Character 40 of Gao and Shubin (2001).
213. Carpals 3 and 4: (0) separate, (1) fused.
214. Prepollex and radiale: (0) separate, (1) fused.
215. Ulnare and intermedium: (0) separate, (1) fused.
216. Ulnare and carpal 4: (0) separate, (1) fused.
217. Intermedium and centrale: (0) separate, (1) fused.
218. Number of centrale in manus: (0) two, (1) one.

Hindlimb

219. Hind limbs: (0) present, (1) absent. Modified from character KK of Duellman and Trueb (1986).
220. Number of toes on hindlimb: (0) five, (1) four, (2) three, (3) two, (4) one. Ordered.
221. Number of phalanges on digit IV of pes: (0) three, (1) four.
222. Phalanges on digit I of pes: (0) two, (1) one.
223. Tibial spur: (0) absent, (1) present, not elongate and pointed, (2) elongate and pointed.
224. Fusion of tarsals 1 and 2: (0) separate, (1) fused. Character 41 of Gao and Shubin (2001).
225. Distal tarsals 4 and 5: (0) separate, (1) fused.
226. Number of centrale in pes: (0) two, (1) one.
227. Cartilaginous element medial to tarsal 5: (0) absent, (1) present.

Vertebral Column

228. Odontoid process of atlas (tuberculum interglenoideum): (0) absent, (1) present. Character JJ of Duellman and Trueb (1986).
229. Odontoid process of atlas, articular surfaces distinct from occipital condyles: (0) no, (1) yes.
230. Atlas, transverse process: (0) absent, (1) present.
231. Trunk region: (0) not elongate, 8–24 presacral vertebrae, (1) elongate, 37–104 presacral vertebrae.
232. Modal presacral number: (a) 8, (b) 9, (c) 10, (d) 11, (e) 12, (f) 13, (g) 14, (h) 15, (i) 16, (j) 17, (k) 18, (l) 19, (m) 20, (n) 21, (o) 22, (p) 23 or more. Ordered. For Bayesian analysis (0) 8–10, (1) 11–13, (2) 14–16, (3) 17–19, (4) 20 and higher.
233. Ribs on mid-body trunk vertebrae: (0) present; (1) absent.
234. Ribs on last trunk vertebra: (0) present, (1) absent.
235. Rib on penultimate trunk vertebra: (0) present, (1) absent.
236. Sacral rib: (0) present, (1) absent.
237. Modal number of caudal vertebrae bearing ribs: (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7, (i) 8, (j) 9, (k) 10. Ordered. For Bayesian analysis: (0) 0–1, (1) 2–3, (2) 4–5, (3) 6–7, (4) 8–10.
238. Presacral ribs at midbody: (0) bicapitate (two processes or distinct articulating surfaces), (1) unicapitate.
239. Dorsal process of bicapitate ribs: (0) articulates with diapophysis, (1) reduced, does not articulate with diapophysis.
240. Cartilage on 4th rib, dorsal and ventral processes, (0) present, (1) absent, one process only or no process.
241. Dorsal process on mid-body of rib of 4th vertebra: (0) absent, (1) present.
242. Sacral rib, dorsal process: (0) absent, (1) present.
243. Bony lamina between diapophyses and paraphyses: (0) absent, (1) present.
244. Bony lamina between ventral and dorsal processes of ribs, (0) absent, (1) present.
245. Paraphyses and diapophyses on mid-trunk vertebra: (0) separated, (1) in contact.
246. Mid-ventral keel on mid-body vertebrae: (0) absent, (1) present.
247. Posterolateral flanges on mid-dorsal keel on mid-body vertebrae: (0) absent, (1) present.
248. Anterior keel on transverse process: (0) absent, (1) present.
249. Anterior process on transverse process (projecting from anterior keel): (0) absent, (1) present, extending beyond anterior margin of centrum.
250. Anterodorsal keel on transverse process (extending from transverse process to anterior zygapophysis): (0) absent, (1) present.
251. Dorsolateral keel on neural arch: (0) absent, (1) present.
252. Paired processes on ventral surface of trunk centra (basapophyses): (0) absent, (1) present at posterior edge of centrum, (2) present at anterior edge of centrum. Unordered.
253. Posteriorly projecting neural spine: (0) present, (1) absent.
254. Neural spine of midbody trunk vertebrae terminates with: (0) paired median processes, (1) single median process.
255. Transverse processes of mid-trunk vertebrae: (0) two, (1) one.
256. Mid-dorsal keel on trunk vertebra: (0) absent, (1) short, (2) raised.
257. Intervertebral bodies of posterior trunk vertebrae: (0) unossified, (1) some or all ossified. Modified from character V of Duellman and Trueb (1986).
258. Caudosacral vertebrae (number of caudal vertebrae lacking a hemal arch, plus the sacral vertebra): (0) 2, (1) 3, (2) 4. Character from Wake (1966). Ordered.
259. Caudal vertebrae, neural spine: (0) with one process, (1) paired processes
260. Mid-dorsal keel on caudal vertebrae: (0) absent, (1) present.
261. Caudal vertebrae, (0) keels absent, low, and/or rounded; (1) dorsal and ventral keels raised and distinctly rectangular.
262. Transverse process of anterior caudal vertebrae: (0) posteriorly oriented, (1) anteriorly oriented.
263. Caudal vertebrae, anterior keel on hemal arch: (0) absent, (1) present.
264. Caudal vertebrae, hemal arch: (0) complete, lateral halves fused to form median process, (1) incomplete, two ventral lamina do not contact or fuse on anterior caudal vertebrae, (2) incomplete for all caudal vertebrae.
265. Caudal vertebrae, hemal arch spine: (0) paired process, (1) single process.
266. Zygapophyses and diapophyses connecting caudal vertebrae: (0) present on all or most vertebrae, (1) absent from posterior caudal vertebrae.

External Characters

267. Skin on dorsal body surfaces: (0) smooth, lacking keratinized spicules, (1) keratinized spicules present.
268. Folds of skin along body: (0) absent, (1) present.
269. External gills: (0) absent, (1) present.
270. Number of gill slits (in adults): (0) none, (1) one, (2) two, (3) three. Modified from character R of Duellman and Trueb (1986). Ordered.
271. Eyelids: (0) present, (1) absent.
272. Costal grooves: (0) absent, (1) present.
273. Lateral line system on head: (0) absent, (1) present.
274. Tips of fingers: (0) not keratinized, (1) keratinized.
275. Gular fold: (0) absent, (1) present.
276. Fleshy fold of skin on lower lip: (0) absent, (1) present.
277. Postorbital groove: (0) absent, (1) present.
278. Nasolabial groove: (0) absent, (1) present. Character LL of Duellman and Trueb (1986).
279. Metatarsal tubercles on hand: (0) absent, (1) present.
280. Fold of skin along posterior margin of limbs: (0) absent, (1) present.
281. Brightly colored stripe on dorsal midline of tail: (0) absent, (1) present.

Literature Characters

282. Spinal nerves in posterior trunk vertebrae: (0) exit intervertebrally, (1) exit intravertebrally. Modified from character X of Duellman and Trueb (1986), data from Edwards (1976).
283. Spinal nerve exit in caudal vertebrae: (0) intervertebral in all caudal vertebrae, (1) intravertebral in some or all caudal vertebrae. Modified from character X of Duellman and Trueb (1986), data from Edwards (1976).
284. Dorsal and ventral roots of spinal nerves in trunk vertebrae: (0) exit through single foramen or interverterbrally in all presacral vertebra, (1) dorsal and ventral roots of second spinal nerve exit through separate foramina, (2) dorsal and ventral roots of all presacral vertebrae exit through separate foramina. Modified from character X of Duellman and Trueb (1986), data from Edwards (1976). Ordered.
285. Basilar papilla: (0) present, (1) absent. Modified from character A of Lombard (1977).

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286. Basilar recessus: (0) present, (1) absent. Modified from character A of Lombard (1977).
287. Recessus amphibiorum: (0) horizontal, (1) vertical. Modified from character B of Lombard (1977).
288. Otic sac: (0) multilobate, (1) bulbar. Modified from character C of Lombard (1977).
289. Otic sac: (0) vascularized, (1) not vascularized. Modified from character C of Lombard (1977).
290. Amphibian periotic canal connective tissue: (0) absent, (1) present. Modified from character D of Lombard (1977).
291. Periotic cistern: (0) large, (1) small. Modified from character E of Lombard (1977).
292. Periotic cistern: (0) not protruding into fenestra ovalis, (1) protruding into fenestra ovalis. Modified from character F of Lombard (1977).
293. Periotic canal joins periotic cistern (0) dorsally at posterior aspect, (1) dorsal and posterior to the fenestra ovalis, (2) by a protrusion of the cistern into the fenestra ovalis. Modified from character G of Lombard (1977). Unordered.
294. Periotic canal: (0) curves ventrally, (1) horizontal. Modified from character H of Lombard (1977).
295. Periotic canal, dorsally directed flexure: (0) absent, (1) one, (2) two. Modified from character H of Lombard (1977). Ordered.
296. Periotic canal, ventrally directed flexure: (0) absent, (1) present. Modified from character H of Lombard (1977).
297. Secondary periotic foramen: (0) present, (1) absent. Modified from character I of Lombard (1977).
298. Recesssus partis amphibiorum: (0) present, (1) absent. Modified from character I of Lombard (1977).

Cloacal Characters (from Sever, 1991)

299. Ciliated epithelium in the cloacal tube of females: (0) present, (1) absent. Character C of Sever (1991).
300. Ciliated epithelium in the cloacal tube of males: (0) present, (1) absent. Character D of Sever (1991).
301. Epidermis in anterior half of female cloacal chamber: (0) absent, (1) present. Character E of Sever (1991).
302. Cloacal recess in females: (0) absent, (1) present. Character F of Sever (1991).
303. Number of pairs of rugae in the male cloaca: (0) <10, (1) >10. Character G of Sever (1991).
304. Primary and secondary folds in the male cloaca: (0) absent, (1) present. Character H of Sever (1991)
305. Dorsolateral recesses in the male cloacal chamber: (0) absent, (1) present. Character J of Sever (1991).
306. Pseudopenis in male cloaca: (0) absent, (1) present. Character K of Sever (1991).
307. Female anterior ventral glands: (0) absent, (1) present. Character L of Sever (1991).
308. Spermathecae: (0) absent, (1) present. Character M of Sever (1991).
309. Spermathecae: (0) not united by a common duct, (1) united by a common duct. Character N of Sever (1991).
310. Female dorsal glands: (0) absent, (1) present. Character O of Sever (1991).
311. Male anterior ventral glands: (0) absent, (1) present. Character Q of Sever (1991).
312. Male posterior ventral glands: (0) absent, (1) present. Character R of Sever (1991).
313. Kingsbury's glands: (0) absent, (1) present. Character S of Sever (1991). Sever's (1991) characters T and W have basically identical distributions to this character, and these characters were not included to avoid possible redundancy.
314. Lateral pelvic glands: (0) absent, (1) present. Character U of Sever (1991).
315. Caudal pelvic glands: (0) absent, (1) present. Character V of Sever (1991).
316. Amphiumid pit glands: (0) absent, (1) present. Character X of Sever (1991).
317. Tubular cloacal glands in females: (0) absent, (1) present. Character P of Sever (1991).

Reproductive and Larval Characters

318. Eggs: (0) laid singly or in clumps, (1) laid in strings. Data summarized by Duellman and Trueb (1986).
319. Eggs: (0) not laid in a spindle-shaped sac, (1) laid in a spindle-shaped sac. Data summarized by Duellman and Trueb (1986).
320. Eggs from each oviduct (0) not compartmentalized, (1) compartmentalized, connected to each other but separated from eggs from other oviduct. Data summarized by Duellman and Trueb (1986).
321. Egg deposition: (0) in water, (1) on land. Data from Duellman and Trueb (1986).
322. Aquatic larvae: (0) present, (1) absent, direct development. Data summarized by Duellman and Trueb (1986).
323. Number of larval gill slits: (0) four, (1) three, (2) two, (3) one. Character R of Duellman and and Trueb (1986), data from these authors. Ordered.
324. Larval balancer: (0) absent, (1) present. Character and data from Crawford and Wake (1998).

Chromosomal Characters

325. Microchromosomes: (0) present, (1) absent, macrochromosomes only, (2) micro and macrochromosomes not distinct, grade into each other. Modified from character DD of Duellman and Trueb (1986), data summarized by these authors. Unordered.
326. Haploid chromosome number: (a) 11, (b) 12, (c) 13, (d) 14, (e) 15, (f) 16, (g) 17, (h) 18, (i) 19, (j) 20, (k) 21, (l) 22, (m) 23, (n) 24, (o) 25, (p) 26, (q) 27, (r) 28, (s) 29, (t) 30, (u) 31, (v) 32, (w) 33. Modified from character DD of Duellman and Trueb (1986), data summarized by these authors. Ordered. For Bayesian analysis: (0) 11–15, (1) 16–20, (2) 21–25, (3) 26–29, (4) 30–33.

APPENDIX 3

Specimens from which RAG-1 sequences were obtained, along with the associated Genbank accession numbers (in parentheses). Institutional abbreviations follow Leviton et al. (1985), with the addition of APSU (Appalachian State University) and KUHE (Kyoto Museum). Abbreviations for field series: ASB (Andrew S. Baldwin), RH (Richard Highton), TCL (Thomas C. LaDuke), TWR (Tod W. Reeder), and WRVD (Wayne R. Van Devender).

Ingroup (Caudata): Ambystomatidae: *Ambystoma gracile*: UTA-A 56595 (AY650131). *Ambystoma opacum*: UTA-A 56611 (AY650130). Amphiumidae: *Amphiuma means*: APSU 23768 (AY650127). *Amphiuma pholeter*: APSU 23767 (AY650128). Cryptobranchidae: *Andrias davidianus*: MVZ 204245 (AY650142). *Cryptobranchus alleganiensis*: TCL 1414 (AY650141). Dicamptodontidae: *Dicamptodon tenebrosus*: UTA-A 56599 (AY650132). Hynobiidae: *Hynobius nebulosus*: KUHE 24698 (AY650144). *Onychodactylus japonicus*: Japan: Tochigi Prefecture: 0.25 mi W Nikko, Japan (D. Weisrock; voucher to be catalogued; AY650143). *Salamandrella keyserlingii*: MVZ 222330 (AY650145). Plethodontidae: *Aneides lugubris*: SDSU 3960 (AY650118). *Batrachoseps major*: TWR 553 (AY650126). *Bolitoglossa helmrichi:* UTA-A 51457 (AY650124). *Desmognathus quadramaculatus*: UTA-A 56601 (AY650117). *Ensatina eschscholtzii*: SDSU 4045 (AY650119). *Eurycea longicauda*: CM 147803 (AY650121). *Eurycea neotenes*: TNHC 60313 (AY650122). *Plethodon elongatus:* RH 75-29 (AY650120). *Pseudotriton ruber*: ASB 130 (AY650123). *Pseudoeurycea rex*: UTA-A 56667 (AY650125). Proteidae: *Necturus beyeri*: WRVD S73 (note that some authorities refer to this population as *"Necturus* sp. cf. *beyeri";* Bart et al., 1997; AY650136). *Proteus anguinus*: Zoological Collection, Department of Biology, University of Ljubljana: Otoski breg #6 (AY650138). Rhyacotritonidae: *Rhyacotriton kezeri*: UTA-A 56614 (AY650129). Salamandridae: *Notophthalmus viridescens*: UTA-A 56597 (AY650134). *Salamandra salamandra*: MVZ 186046 (AY650135). *Taricha rivularis*: UTA-A 56597 (AY650133). Sirenidae: *Pseudobranchus axanthus*: UTA-A 56600 (AY650139). *Siren intermedia*: UTA-A 56617 (AY650140).

Outgroups: Anura: Ascaphidae: *Ascaphus montanus*: APSU 24182 (AY650146). Gymnophiona: Caeciliidae: *Dermophis mexicanus*: UTA-A 51487 (AY650148). Ichthyophiidae: *Ichthyophis* sp. (Sumatra): UTA-A 55276 (AY650147).

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J Mol Med (2007) 85:317–329
DOI 10.1007/s00109-006-0143-4

REVIEW

Multifunctional antimicrobial peptides: therapeutic targets in several human diseases

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Received: 6 August 2006 / Revised: 10 October 2006 / Accepted: 10 November 2006 / Published online: 10 January 2007
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Abstract Antimicrobial peptides have emerged as promising agents against antibiotic-resistant pathogens. They represent essential components of the innate immunity and permit humans to resist infection by microbes. These gene-encoded peptides are found mainly in phagocytes and epithelial cells, showing a direct activity against a wide range of microorganisms. Their role has now broadened from that of simply endogenous antibiotics to multifunctional mediators, and their antimicrobial activity is probably not the only primary function. Although antimicrobial peptide deficiency, dysregulation, or overproduction is not known to be a direct cause of any single human disease, numerous studies have now provided compelling evidence for their involvement in the complex network of immune responses and inflammatory diseases, thereby influencing diverse processes including cytokine release, chemotaxis, angiogenesis, wound repair, and adaptive immune induction. The purpose of this review is to highlight recent literature, showing that antimicrobial peptides are associated with several human conditions including infectious and inflammatory diseases, and to discuss current clinical development of peptide-based therapeutics for future use.

Keywords Antimicrobial peptides · Inflammatory diseases · Innate immune system · Infection · Host defense

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Introduction

Antimicrobial peptides (AMPs), also known as genetically encoded antibiotic peptides, are conserved components of the innate immune response [1, 2]. They are produced in many organisms including bacteria, insects, plants, and vertebrates, where they represent a defense system [3–5]. In mammals, these peptides function mainly in phagocytic cells of the immune system to kill engulfed or invasive bacteria and in mucosal epithelial cells to prevent colonization of host tissues by pathogens [1, 6]. In the past two decades, numerous antimicrobial peptide families from many living organisms have been described. In humans, three distinct groups were characterized, including defensins, cathelicidins, and histatins. Additional peptides that have been identified include RNase 7, granulysin, hepcidin, dermacidin, and lactoferrin. As of 2006, the inventory of AMPs comprises about 900 peptides of different origins, and these peptides have been listed in a database in Trieste (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). Additional information with

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regard to these peptides is also available from another website: http://aps.unmc.edu/AP/main.htm. Medline database search using the PubMed interface with "antimicrobial peptides" as key word also shows more than 7,000 references with new references added every week.

AMPs have potent activities against a broad range of microorganisms that covers gram-positive and -negative bacteria, fungi, parasites, and enveloped viruses [7, 8]. Several excellent review articles have examined the role of these peptides in the protection of a variety of human tissues [9–11]. The precise details of their mechanism of action remain unknown. However, it is generally accepted that the positively charged peptides act directly on the negatively charged cellular membranes of bacterial cells, causing an increase in membrane permeability, which then leads to rapid cell death [12, 13]. Other mechanisms of action, including activity on intracellular targets after membrane permeabilization, have been proposed. Examples of intracellular activity include the activation of autolytic enzymes and the inhibition of DNA and protein synthesis [13, 14]. Buforin II has been shown to enter the cell and accumulates in the bacterial cytoplasm where it inhibits cellular functions by binding to DNA and RNA [15]. Attacins block the synthesis of integral membrane proteins [16]. PR-39 was reported to inhibit DNA synthesis [17]. Interestingly, pyrrhocoricin, an insect peptide, was demonstrated to kill bacteria by binding to a protein target called DnaK [18], thereby preventing it from performing its protein-repair function, leading to death of the bacteria. Recently, a different mechanism has been proposed for the θ-defensin, retrocyclin 2, which blocks influenza virus infection by cross-linking and immobilizing surface glycoproteins, resulting in the inhibition of viral entry into the cell [19]. Together, these data make AMPs a very attractive target for clinical development to treat conditions where traditional antibiotics are becoming less effective. As AMP research has proliferated, the repertoire of biological roles of AMPs has expanded to a number of other functions (Fig. 1). These include (1) endotoxin neutralization, a property that allows detoxification of LPS [20], (2) chemokine-like activity, based on chemokine structural motif similarity that allows AMPs such as β-defensins to interact with certain chemokine receptors [21], and (3) immunomodulatory activities linking innate to adaptive immune responses [22, 23].

More recently, there has been growing interest in the role of these peptides in several inflammatory conditions including psoriasis, arthritis, respiratory diseases, atherosclerosis, and wound repair. These novel findings will be summarized in this review. It is hoped that a better understanding of how AMPs contribute to these diseases will provide important clues to the "patho-etiologies" of many of these complex conditions that have often been labeled as idiopathic when no cause is known. These researches would pave the way for the development of novel therapeutic drugs.

Antimicrobial peptides against infectious diseases

Why AMPs? Although the discovery and the development of antibiotics during the mid-twentieth century provided potent antimicrobial drugs with high specificity, increasing antibiotic resistance in microorganisms and new emerging pathogens have become a major problem in our society [24]. Several human pathogens are now becoming resistant to a number of clinically significant antibiotics, causing a crisis in the treatment and management of infectious diseases. Strains of *Mycobacterium tuberculosis*, *Enterococcus faecium*, and *Pseudomonas aeruginosa* are no longer easily treated with antibiotics, and increased resistance is observed in other important human pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. The resistance phenomenon has been attributed to the overuse of antibiotics and the increasing number of immunocompromised patients who have alterations in phagocytic, cellular, or humoral immunity. The diminished effectiveness of current therapies and the widespread resistance has prompted interest in the search for new antibiotics as alternative treatments. A developing area of study using natural peptides with antimicrobial activity may offer the solution for the encountered resistance and hold promise for development of new therapeutic agents.

Evidences for antimicrobial activity of natural peptides

A growing body of evidence has demonstrated that AMPs work as antibiotics. Data from in vitro as well as from in vivo models have provided compelling evidence that these peptides protect against a range of microorganisms including bacteria, enveloped viruses, fungi, and even certain parasites.

AMPs against bacteria Direct antibacterial activity of AMPs has been demonstrated by a number of studies. Depleted levels of antibiotic peptides have been shown to be associated with several pathologic conditions. For instance, in humans, patients with a specific granule deficiency syndrome lack α-defensins and suffer from severe and frequent bacterial infections [25]. Others with a condition known as morbus Kostmann suffer from frequent oral bacterial infections and severe periodontal disease, which correlates with a deficiency in the human cathelicidin peptide LL-37 and human neutrophil peptides (HNP1-3) [26]. Low expression of LL-37, human β-defensin (hBD)-2, and hBD-3 in skin lesions caused by atopic dermatitis coincides with enhanced susceptibility to

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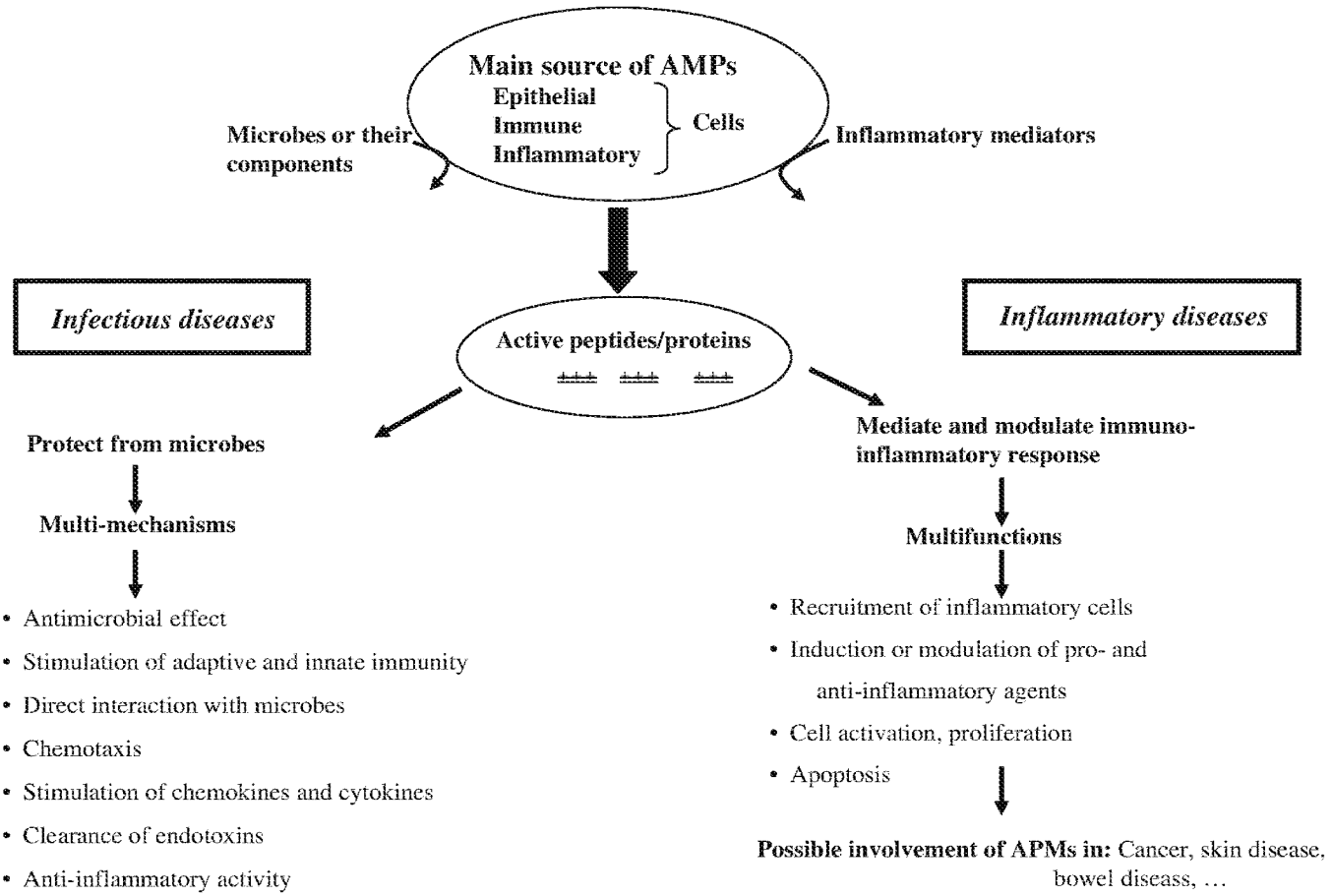


Fig. 1 Multifunctions of antimicrobial peptides in infectious and inflammatory diseases. After their release from various cell types after activation by microbes or inflammatory mediators, AMPs show a number of functions, including antimicrobial activity and modulation of the inflammatory response in different inflammatory diseases. Primary potential mechanisms of action of these peptides are *highlighted*

skin infections [27]. In rodent models, cathelicidins can control bacterial load and prevent mortality when administered after bacterial challenge [28, 29]. β-defensin-1 and CRAMP (*cnlp*) gene in knockout mice are more susceptible to and fail to clear infections [30, 31]. Conversely, overexpression of human defensins protected mice against enteric salmonellosis [32]. Systemic administration of nisin, a lanthionine-containing peptide from *Lactococcus lactis*, protected mice against *S. pneumoniae* infection [33]. The antibiotic nature of AMPs was also confirmed by a recent study demonstrating that in transgenic mice, overexpression of the porcine cathelicidin PR-39 enhanced resistance and protection of these animals against group A *Streptococcus* [34]. Lately, a fungal peptide named plectasin, belonging to the family of defensins, has shown antibacterial activity particularly against *S. pneumoniae*, including strains resistant to conventional antibiotics [35].

Although genetically modified mice contribute significantly to the studies of AMPs in vivo, evaluation of how specific peptides contribute to the host response after pathogenic challenge is complicated by a number of issues including (a) presence of large peptide families including 22 alpha-defensins in mice or 35 beta-defensins in humans may lead to functional redundancy, (b) other proteins such as lysozyme and lactoferrin also contribute to host defense, and (c) many AMPs have overlapping antimicrobial activity against gram-positive and gram-negative bacteria. Therefore, to identify the unique contribution of each AMP to disease, specific AMPs in knockout mice and relevant disease models will need to be studied.

Another method used to demonstrate the antibiotic properties of these peptides consisted of gene therapy approach in which mice were inoculated with an adenovirus vector containing the DNA for the human peptide LL-37 [36]. Success of this approach was corroborated by demonstration that overexpression of porcine PR-39 or human LL-37 in keratinocytes transduced with a recombinant lentivirus enhanced bacterial killing [34, 37]. AMP cutaneous gene therapy has also been successfully used in infected wounds and burns [38, 39]. However, although the concept of developing AMPs as antibiotics appears promising, concerns about bacterial resistance to these peptides

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have been raised. Experimental analysis has identified a number of mechanisms of bacterial resistance to AMPs including altered cell surface charge, active efflux, production of proteases or trapping proteins, and modification of host cellular processes (reviewed in [40, 41]). Therefore, knowledge of the molecular basis of bacterial resistance to AMPs may provide clues on how to circumvent potential resistance problems. This research will build a basis for the development of AMPs as an alternative therapy to conventional antibiotic agents for the treatment of drug-resistant pathogens.

AMPs against viruses Apart from the antibacterial activity, AMPs such as defensins also possess antiviral activity. Based on the pattern of cysteine connectivity, mammalian defensins are classified into alpha and beta families. Recently, a circular form of AMPs called θ-defensin has been characterized in macaques, whereas in humans, the gene coding this peptide had been silenced by mutation [42]. Different studies have reported that defensins inhibit viral replication [43, 44]. For example, the α-defensins target the human immunodeficiency virus (HIV) activity by directly inactivating viral particles and affecting the ability of the virus to replicate within CD4 cells [45]. Human α-defensins HNP-1 to -3 and HD-5 have been shown to block papillomavirus infection [46]. Retrocyclin 2, a synthetic θ-defensin peptide that humans do not synthesize due to a mutation in the corresponding human gene, has the capacity to block influenza virus infection [19]. Human β-defensins can also block HIV-1 replication, and interestingly, a single-nucleotide polymorphism in a β-defensin gene has been associated with clinical manifestation of HIV-1 infection, suggesting that the human β-defensins play an important role in host defense against HIV [47]. Cathelicidins, in contrast, have an inhibitory effect on lentiviral replication in vitro [48], and LL-37 appears capable of interfering with vaccinia virus replication in vitro and in mice [49]. Dermaseptin S4, a 28-residue AMP isolated from frog skin, attenuates HIV infection in vitro [50]. Other AMPs from frog skin including caerin 1.1, caerin 1.9, and maculatin 1.1 have also demonstrated inhibition of HIV in vitro [48].

How AMPs inhibit viral entry into cells? HIV entry into a target cell is facilitated by its gp120/gp41 glycoprotein (env) interaction with CD14 [51] and a coreceptor, usually CCR5 or CXCR4 [52]. Generally, defensins inhibit HIV entry into the cell by inhibiting one of these mechanisms. For instance, θ-defensin has been shown to prevent HIV-1 env-mediated fusion by binding gp41 and blocking its 6-helix bundle formation [53]. hBD-3 has been reported to inhibit HIV infection by competing with stromal-derived factor, the natural ligand for CXCR4 [54]. Therefore, AMPs, in particular defensins, have clear anti-HIV-1 activity, and their broader anti-viral functions await further exploration.

AMPs against fungi The increasing incidence and severity of invasive mycoses and the resistance of fungal pathogens to currently available antifungal drugs have led scientists to explore the antifungal properties of AMPs. To date, approximately 100 peptides have been investigated for their capacity to counteract fungal infections, particularly those caused by *Candida spp.* [55]. *Candida spp.* have emerged as the fourth most common cause of bloodstream infections in the USA. Human HNP-1 and HNP-2 have shown fungicidal activity against *Candida albicans* and inhibited significantly the growth of *Cryptococcus neoformans* [56]. Rabbit NP-1, NP-2, and NP-3 also demonstrated good efficacy against *C. albicans* [57]. Histatins are another family of peptides with antifungal activity that are selectively secreted by the human parotid and submandibular glands. Among these peptides, histatins 1, 3 and 5 have been identified and characterized earlier, and their differential fungicidal activity against *C. albicans* has been determined [58]. Additional evidence for the role of AMPs in the protection against fungi has come from a study showing that low levels of histatins from saliva in a group of HIV patients correlated with higher incidence of oral candidiasis [59]. Interestingly, not all strains of *Candida* are sensitive to the action of naturally occurring AMPs. A recent study reported that *Candida glabrata* shows resistance to histatins as well as to magainins [60]. The major mechanism(s) of action by which these peptides inhibit fungi is not clear yet. However, several modes of action have been proposed including binding to and disruption of the outer membrane [61], resulting in leakage of important intracellular contents, and interaction with specific internal targets once they penetrate the cell [62]. Together, these studies have provided evidence that cationic peptides and particularly histatins may represent a new generation of compounds for the treatment of oral fungal infections.

AMPs against protozoa Early studies have demonstrated antiprotozoan activity of AMPs [63, 64]. Since then, considerable research efforts have been directed toward understanding the activities of these peptides against parasites transmitted by insect vectors. Recently, the potential activities of defensins and cathelicidins against the African trypanosome *Trypanosoma brucei* have been explored [65]. *T. brucei* is the vector-borne protozoan parasite that causes sleeping sickness, a disease associated with significant morbidity and mortality in both humans and animals. This study clearly demonstrated the effectiveness of peptides against parasites by disruption of their cell membrane integrity. Further investigation has shown that administration of cathelicidin AMPs to mice with late-stage *T. brucei*

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infection rapidly improved parasitemia and prolonged survival [65]. These studies provide evidence for the use of AMPs in the treatment of parasitic diseases and invite more research that examines the role of AMPs in the control of parasitic infections.

Antimicrobial peptides and inflammatory diseases

A number of studies have suggested an association between the activation of innate immune mechanisms and the pathogenesis of inflammatory diseases. Recruitment of inflammatory cells and accumulation of chemokines and proinflammatory cytokines are hallmarks of an inflammatory state. Immune cell infiltrates are the primary source of host antimicrobial agents including AMPs, and released AMPs serve to amplify inflammation. In this context, different studies reported that AMPs including LL-37 may be involved in the regulation of inflammation by activating chemokine release from different cell types [66–68].

Apart from a central role in prevention and clearance of infections, AMPs exhibit an array of ever-expanding functions [69, 70]. The importance of AMPs in inflammatory conditions has now been expanded to include the following: psoriasis, respiratory disorder, inflammatory lung disease, inflammatory bowel disease (IBD), rheumatoid arthritis, and atherosclerosis (Table 1). The potential implication of a role of natural peptides in these diseases will be the major focus of our next discussion.

AMPs and skin diseases

Psoriasis is a common immune-mediated chronic skin disease that comes in different forms and differing levels of severity. Study of psoriatic-scale extracts revealed the presence of several AMPs including LL-37, human neutrophil defensin 1-3, RNase 7, and lysozyme (reviewed in [71, 72]). These peptides are believed to protect inflamed skin from microbial infections, as evidenced by the fact that patients who have psoriasis rarely suffer from skin infections, whereas patients with atopic dermatitis have increased skin infection rate with *S. aureus* [27]. In line with this observation, there exists a good correlation between atopic dermatitis and an impaired innate defense of human skin [73]. High-level expression of AMPs is seen in other pathologic conditions. For instance, hBD-2 and HNP are abundant in lesions of superficial folliculitis, a common skin disease characterized by inflammation of the hair follicle and infection with *S. aureus* [74]. Furthermore, human LL-37 has been shown to be induced in systemic lupus erythematosus and contact dermatitis [75]. From these diverse studies, it is becoming clear that AMPs are involved in skin diseases. However, their role in pathophysiology of inflamed skin tissues is not fully understood. Therefore, future studies must focus on the purported dual functions of AMPS, namely, its antimicrobial activity and its modulation of inflammatory response.

Acne vulgaris is a chronic inflammatory disorder of the pilosebaceous unit that widely affects adolescents and young adults (reviewed in [76]). The main events in the development of an inflammatory acne vulgaris lesion involve *Propionibacterium acnes* colonization and proliferation, which has shown resistance to conventional antibiotics. Because of their ability to neutralize endotoxins and consequently to inhibit the secretion of proinflammatory cytokines (e.g., TNF-α, IL-1) by host cells, cationic peptides could be potential candidates for treatment of acne. Granulysin is an antimicrobial peptide of the saponin-like family reported to be present in acne lesions. Recently, it has been reported that granulysin-derived peptides kill *P. acnes* effectively [77] and possess anti-inflammatory effects, as demonstrated by the suppression of *P. acnes*-stimulated cytokine release. Study of another peptide designated MX-594AN, which is under clinical development by Migenix, has demonstrated significant decrease in lesion counts of acne vulgaris in humans [78]. Taken together, these studies suggest that AMPs may be useful as agents for the treatment of acne. Hence, development of peptide-based drugs, preferentially applied topically, should be one area of research focus to resolve skin infections such as acne infection and other worse conditions.

AMPs and respiratory and lung disorder

Respiratory secretions have microbicidal properties mediated by their constituent antimicrobial peptides/proteins including lysozyme, lactoferrin, secretory leukocyte protease inhibitor (SLPI), defensins, and cathelicidins. These molecules are produced by airway epithelial and inflammatory cells and are involved in many processes such as host defense, stimulation of adaptive immune response, and modulation of inflammatory response of lung disease [79]. Evidence for their role in lung disorder came from a number of recent studies describing the alteration of antimicrobial concentration in several pulmonary disease states [80]. Increased defensin levels were observed in cystic fibrosis, diffuse panbroncheolitis, idiopathic pulmonary fibrosis, acute respiratory distress syndrome, and lung transplant [81, 82]. Cystic fibrosis, a genetic disease associated with recurrent bacterial infections of the airways and inflammation, represents one illustration of the implication of AMPs in human pathologies. The defective chloride channel causing the disease increases salinity of the alveolar fluid and thus impairs the bactericidal activity against *P. aeruginosa* of β-defensins, which are salt

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Table 1 Expression level and proposed functions of antimicrobial peptides/proteins in various human inflammatory diseases

Disease state	Peptides	Expression levels and potential functions
Skin inflammatory diseases		
Psoriasis	LL-37, defensins	Overexpressed, absence of *S. aureus* [27]
Atopic dermatitis	LL-37, defensins	Downregulated, presence of *S. aureus* [27]
Lupus, erythrematous, and contact dermatitis	LL-37	Increased [75]
Acne vulgaris	MX-594 AN	Inhibits *P. acne* [78]
	Granulysin	Kills *P. acne*, anti-inflammatory action [77]
Respiratory diseases		
Cystic fibrosis	LL-37, β-defensins	Reduced antimicrobial activity due to salt accumulation [7]
Periodontal disease	Defensins	Reduced in saliva of patients with oral candidiasis [142]
	LL-37	Absent in patients with congenital neutropenia [26]
	Histatin 5	Protects periodontium from bacterial infection [89] and prevents biofilm formation [90]
Inflammatory bowel disease		
Crohn's disease	HD5 and HD6	Deficient expression in Paneth cells
	HD5 and HD6	Reduced in CD patients with Nod2 mutation [91]
	LL-37	Expression is altered [91]
Ulcerative colitis	HD5, 6; hBD2–4	Upregulated in patients with UC [95]
Cancer	Magainin II	Toxic effect against cancer cell lines melanoma, breast and lung cancer, lymphoma, and leukemia [97, 98]
	Insect cecropins	Lyse tumour cells [100]
	Bovine lactoferrin	Inhibits lung and liver metastasis of murine melanomas and lymphomas [101] and cytotoxic toward neuroblastoma cells [103]
Atherosclerosis	Defensins	Involved in lipoprotein metabolism [111, 113], exhibit antifibrolytic activity [111], and regulate angiogenesis [115]
	LL-37	Increased expression in human lesions [118]
Inflammatory articular joints	hBD-3, LL-37	Upregulated in osteoarthritis [124]

sensitive [83]. In this context, overexpression of human LL-37 in lungs of mice inhibited bacterial load and inflammatory response after pulmonary challenge with *P. aeruginosa* [84]. In a further investigation, gene transfer approach restored the expression of LL-37 in a cystic fibrosis xenograft model and restored bacterial killing [85]. Overall, these peptides/proteins act as the first line of defense against pulmonary infection and may amplify the adaptive immune response by inducing the production of cytokines, which promote T cell-dependent cellular immunity and antigen-specific Ig production [86], or by enhancing both cellular (Th1-dependent) and humoral (Th2-dependent) cytokine production and immune responses [87]. Future studies should identify AMPS active against cystic fibrosis-associated pathogens including *P. aeruginosa*, *S. aureus*, and *Burkholderia cepacia*. Additionally, these peptides need to be selected or engineered to retain their activities at a high salt concentration.

AMPs and periodontal disease

Human periodontitis is a multifactorial inflammatory oral disease with a complex pathogenesis. It is initiated by a group of predominantly gram-negative anaerobic bacteria such as *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Actinobacillus actinomycetemcomitans*. One primary barrier to infection is the oral mucosa with its innate and adaptive immune processes. Disruption of this tissue can lead to infection by a variety of microbial challenges that can cause periodontal diseases. In line with this observation, patients with a severe congenital neutropenia have AMP deficiency, which is associated with the occurrence of infection and periodontal diseases [26]. A recent study has shown the expression of hBD-1 and hBD-2 peptides in gingival epithelia from periodontally healthy subjects, as well as patients with unsolved chronic periodontitis, suggesting a role of these peptides in periodontal health and disease [88]. Histatin 5, a salivary peptide, was reported to protect the periodontium from host and bacterial enzymes implicated in periodontal disease [89]. More recently, histatin 5 was shown to prevent biofilm formation, thereby reducing denture-induced stomatitis [90]. Thus, salivary AMPs could play a primary role in blocking of periodontal disease initiated by microbes. The knowledge gained from these and future studies will help identify the role of AMPs in periodontal pathogenesis and will provide support for the ongoing attempts to develop these peptides as drugs for the prevention of oral diseases.

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AMPs and chronic inflammatory bowel diseases

In the small intestine, Paneth cells are the major source of AMPs that purportedly play a crucial role in intestinal immunity. A breakdown of this functional barrier may lead to aberrant production of defense molecules and consequently to IBD. IBD is a chronic inflammation of the intestinal mucosa often grouped into two major entities: Crohn's disease (CD) and ulcerative colitis (UC).

Crohn's disease is attributed in part to intestinal bacteria that may initiate and perpetuate mucosal inflammation in genetically susceptible individuals [91]. Studies of transgenic and knockout mice support a pivotal role of Paneth cell α-defensins in protection from bacterial pathogens. New data suggest that deficient expression of Paneth cell α-defensins (HD5 and HD6) may contribute to the pathophysiology of CD [91, 92]. These observations are supported by recent findings showing that mice lacking Nod2, an intracellular sensor of bacteria-derived muramyl dipeptide, fail to express cryptidins, equivalents of human α-defensins [93]. Similarly, human α-defensin expression is diminished in CD patients, particularly in those with Nod2 mutations [91]. Besides defensins, cathelicidin peptides also showed alteration of their expression profile in IBD [94].

Ulcerative colitis is considered a consequence of a failing adaptive and/or innate immune system, in particular AMPs, to cope with pathogenic microorganisms in the intestine. Its has been demonstrated that the expression of hBD-2 to -4 is upregulated in colonic enterocytes in patients with UC [95] and that HD5, HD6, and lysozyme are overexpressed due to metaplastic Paneth cell differentiation in UC colon [96]. Overall, these data provide compelling evidence supporting the hypothesis that AMPs play an important role in intestinal defense, and reduction in their expression may compromise host defense and initiate inflammatory diseases. The discovery of factors such as Nod2 [93] that have a direct impact on the expression of AMPs may contribute to development of future preventive and treatment strategies against IBD.

AMPs and cancer

Cancer treatment using classical chemotherapy approach presents a number of limitations, including toxicity and development of multi-drug resistance by cancer cells. Several reports have demonstrated that AMPs are emerging as a promising class of new natural drugs with toxic activity towards cancer cells. In this aspect, magainin II was shown to exert cytotoxic effect against a wide range of cancer cell lines including melanoma, breast and lung cancers, as well as lymphomas and leukemias [97, 98]. Further studies carried out in vivo have shown that magainin peptides improve survival of animals with ascites-producing tumors [97]. More recently, magainin II was reported to exert cytotoxic and antiproliferative efficacy by pore formation in bladder cancer cells but had no effect on normal murine or human fibroblasts [99]. Cecropins, insect-derived cationic peptides, were also found to be effective in lysis of tumor cells including multidrug-resitant tumor cell lines [100]. In contrast, bovine lactoferricin inhibited liver and lung metastasis of both murine melanomas and lymphomas [101] and induced apoptosis in human leukaemia and carcinoma cell lines [102]. In another study, Eliassen et al. [103] reported that lactoferricin B shows cytotoxicity to neuroblastoma cells in vitro and inhibits the growth of neuroblastoma xenograft in nude rats. Interestingly, lactoferricin seems to induce a rapid destabilization of tumor cell cytoplasmic membrane, triggering apoptosis cascades at a dose that does not affect normal fibroblasts or erythrocytes [104]. Moreover, α-defensins were localized to solid epithelial tumors, specifically oral squamous cell carcinoma, lung tumors, and renal cell tumors, suggesting an association of these peptides with cancer development [105–107]. Recently, an association between the overexpression of α-defensin peptides and bladder cancer invasiveness was reported [108]. However, a physiological approach involving animal models will help to better define the biological activity of these peptides toward cancer cells and to clarify the molecular mechanism by which these cells become susceptible to some of the AMPs. So far, at least three mechanisms have been proposed, (1) cell membrane lytic effect, (2) activation of intrinsic pathways of apoptosis via mitochondrial membrane disruption (reviewed in [109]), and (3) certain peptides are potent inhibitors of blood vessel development that is associated with tumor progression; extensive studies need to be performed to prove that these peptides do not destroy vital organs and have lower toxicity toward normal cells. If all these desirable properties are proven, then AMPs could be the next generation of drugs for cancer therapy. Hence, the development of a strategy that can be used to efficiently deliver apoptosis-promoting and/or anti-angiogenic peptides to both tumor cells and tumor vasculature should be another issue to take in consideration.

AMPs and the inflammatory process of atherosclerosis

Atherosclerosis is a complex and chronic inflammatory disease process characterized by the retention and modification of low-density lipoproteins within the wall of blood vessels [110] and the development of local inflammation event. AMPs produced by endothelial cells and neutrophils are present within atheromatous plaques in the cerebral and coronary circulation [111, 112]. The potential role of these peptides in the development of atherosclerosis, including

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endothelium dysfunction, has been investigated. In this context, defensins were found to have multiple functions. They participate in the lipoprotein uptake and degradation in the vessel wall [111, 113]. They possess antifibrolytic activity [114] and regulate angiogenesis [115, 116]. Lately, animal studies have shown that neutrophil α-defensins cause endothelial cell dysfunction in porcine coronary arteries by reducing endothelium-dependent vasorelaxation [117]. Besides defensins, cathelicidins have also been investigated in the atherosclerosis development process. A sixfold increase in LL-37 transcripts in human atherosclerotic lesions compared with normal arteries was reported [118]. Using gene transfer approach, adenoviral PR-39 was reported to improve blood flow and myocardial function in a pig model of chronic myocardial ischemia by enhancing collateral formation [119]. Antimicrobial peptides/proteins have also been associated with an emerging global problem of obesity and diabetes, a complication that leads to increased burden of heart disease. In diabetic patients, an interesting theory on lactoferrin and lysozyme inactivation proposes specific binding of these proteins to glucose-modified proteins bearing advanced glycation end products [120]. These motifs inhibit endogenous bacterial proteins, thereby increasing susceptibility to bacterial infections in the diabetic population. However, a number of questions have not been answered convincingly. For instance, what is the biological significance of these findings? Is increased expression of these peptides beneficial or harmful to the vessel wall? Is there a link between AMP expression and modified lipids known as a major risk factor for the development of atherosclerosis? Because of the high prevalence of atherosclerosis and the resulting complications, clinical studies are needed to explain the significance of the above-mentioned findings.

AMPs and articular joints

For the last couple of years, AMPs have been investigated in articular joint inflammation including pyogenic arthritis, rheumatoid arthritis, and osteoarthritis. Their presence in human articular cartilage has been reported previously [121]. Further studies have revealed an alteration in the expression of these peptides in synovial membrane from patients with inflammatory joint disease [122], suggesting a role in the protection against microbial infection. However, the upregulation of hBD-3 and LL-37 in osteoarthritis without bacterial challenge has led scientists to believe that they have functions other than the ability to kill microbes. This hypothesis was confirmed later by experiments using animal model for osteoarthritis showing an induction of mouse AMPs in the pathogenesis of osteoarthritis in vivo [123, 124]. Of interest is the finding of a report by Varoga and his colleagues [125], showing overexpression of hBD-3 in osteoarthritis cartilage without bacterial challenge. The observed induction of defensins was attributed to their modulation by proinflammatory cytokines including TNF-alpha and IL-1, known to regulate AMPs [126, 127]. Together, these findings widen our knowledge of the functional spectrum of AMPs, particularly that of hBD-3, which is a multifunctional peptide with the ability to link host defense mechanisms and inflammation with tissue-remodeling processes in articular cartilage. A full knowledge of the exact function of these intrinsic peptides in articular cartilage and synovial membrane will provide further insight into prevention of human articular joint disease.

AMPs and injury, angiogenesis and wound healing

Angiogenesis is one of the complex biological processes of inflammation and wound repair [128]. For instance, when skin epithelial barrier function is breached as a result of injury, wound repair process requires both recruitment and coordination of numerous cell types including inflammatory cells. Neutrophils and macrophages are among cells that invade wound areas and constitute the major source of several angiogenic growth factors and AMPs such as LL-37, which exerts chemoattractant and angiogenic activity. In fact, the expression of LL-37 was reported to be increased in wounded skin [129]. The biological significance of this alteration is probably the protection of injured tissue until it is restored. Growth factors are of major importance in wound healing. Insulin-like growth factor I and TGF-alpha have been shown to induce the expression of the antimicrobial protein hCAP-18/LL-37 (human cationic antimicrobial protein of 18 kDa), hβD-3, neutrophil gelatinase-associated lipocalin, and SLPI in human keratinocytes [130]. In this context, mice deficient in SPLI have a deficiency in cutaneous wound repair that is associated with increased inflammation [131]. Another compelling evidence of the role of AMPs in wound repair comes from a study by Heilborn and his colleagues [132] showing that LL-37 peptide is involved in the re-epithelialization of human skin wound, and its reduction is associated with chronic ulcer epithelium. Another potential effect of AMPs is seen in burn wounds. In severely burned patients, there is a relative deficiency in certain AMPs, and these patients have greater risk of infection [133, 134]. A study by Steinstraesser et al. [135] investigated the in vivo antimicrobial activity of protegrin-1 using an animal burn model. This study showed a rapid decrease in bacterial counts compared to the controls infected in burn wounds.

Using in vitro and in vivo models for wound healing, Koczulla et al. [136] have identified another novel biologic activity of human LL-37, which consists of induction of angiogenesis and arteriogenesis. Interestingly, the porcine cathelicidin PR-39, has been shown to induce a robust

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angiogenic response in in vivo and in vitro experimental models [137]. In conclusion, the multiple links among AMP expression, increased angiogenesis, and tissue repair involve a number of pathways, including those of toll-like receptors, inflammatory cytokines, and putative "danger" signals. Together, these complicated processes could have several implications, including protection of vulnerable tissue against infection, growth promoting activities, and epithelialization events.

Clinical development of antimicrobial peptides

The challenge at hand is to use current knowledge of AMPs in the design and development of useful drugs. In this respect, biotechnology firms have begun development of new peptide-based compounds that show different and potentially improved resistance profiles compared to early antibiotics. A comprehensive summary of AMP-based therapeutics under development has been published recently [11]. Representative examples of peptides going preclinical or clinical trials are discussed next:

- *Plectasin* (Novozymes) is a defensin peptide that shows good microbicidal activity against antibiotic-resistant bacteria such as those responsible for diseases like pneumonia [35]. Unlike other clinically tested AMPs, plectasin appears to be tolerated at high doses and shows efficacy in treatment of systemic infections. The development of plectasin as a therapeutic agent is currently entering preclinical phase with the anticipation that a product will be approved for clinical use in 8–10 years.
- *P-113* (Dermegen) is a 12-amino acid fragment derived from histatin 5 with anti-candidal activity comparable to that of the parent form. This peptide is in phase I/II clinical trials and is used as mouth rinse for treatment of plaques and gingivitis. Data from a human experimental model showed that P-113 is active against gingivitis and plaques [138].
- *MBI-226* (Migenix) is an Idolicidin analogue in phase III clinical trials. It will be used for the treatment of catheter-related blood stream infection [139].
- *MX-594AN* (Migenix) is an antimicrobial cationic peptide in development that has been formulated as topical treatment for mild to moderate acne vulgaris. MX-594AN is under clinical trials and appears to demonstrate efficacy against all types of acne lesion. Migenix has also developed MX-594AN under the name CLS001, which is tested for topical treatment of rosacea. Phase II trials are due to be completed in 2007.
- *PG-1 protegrin* (Intrabiotics) is a peptide in phase III clinical trial designed for the treatment of peritoneal infections caused by *P. aeruginosa, S. aureus*, and methicillin-resistant *S. aureus*.
- *rBPI-21* (Xoma) is a recombinant form of human bactericidal/permeability-increasing (BPI) protein that has reached phase II/III clinical trials and will be used for treatment of meningococcaemia and Crohn's disease.
- *Heliomycin* (EntoMed) is a peptide derived from insects with antifungal activity, currently in preclinical trials.

In summary, although several clinical trials underlined the broad therapeutic potential of AMP-based drugs, these agents are still at an early stage of technologic maturation, and many hurdles have yet to be overcome.

Future perspectives

Although no peptide has yet reached the drug market, pharmaceutical companies remain enthusiastic about the prospect of developing these novel agents as a new generation of medications. Additionally, researchers are now convinced that these natural peptides have functions beyond those of antimicrobial activities and represent an attractive target for the future design of therapeutic agents. The previous section has provided examples of the promising use of AMPs in the clinical setting. However, there are a few drawbacks to consider. As AMPs are of peptidic nature, they could present the following problems: (1) high manufacturing costs, (2) short half life, (3) lost of activity in physiological conditions [140], (4) application problems, (5) unwanted systemic reactions (aggregation, half-life, or immunoreactivity), and (6) interference with normal flora bacteria that may arise when trying to use those peptides as antibacterial agents [141]. Additionally, there remains a number of unresolved issues to consider: (1) Standardized techniques to assess the activity of these peptides is still unavailable. (2) Molecular regulation mechanisms of certain alpha-defensin genes are poorly defined. Detailed knowledge of how AMPs are regulated will allow researchers to create drugs that directly modulate the expression of specific AMPs to ameliorate the specific disease condition. 3) The ability to target these future medications to the site of disease remains a challenge. AMP gene therapy could be a promising approach to revolutionize treatment of many inflammatory and infectious diseases once strategies such as gene delivery are optimized. In this sense, one of the goals will be to design a system that simultaneously achieves high efficiency, prolonged gene expression, and low toxicity. However, as it was mentioned above, formidable challenges related to AMP properties are still to be faced. 4) Tolerance and toxicity issues of these

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peptides need to be addressed, especially as their cationic property make them prone to interact with anionic components of the host cells. An ideal peptide drug would be one that can be tolerated at high dose, resists degradation, and is suitable for topical as well as intravenous administration. 5) Finally, understanding of the role and expression of AMPs both in health and disease remains a challenging area of research.

In conclusion, research that focuses on the above issues should provide further important insights into the pathophysiological roles AMPs play in complex diseases. This knowledge will lay the foundation for the use of these peptides as prototypes of innovative drugs and/or as potential sensors and biomarkers for early detection and prevention of diseases.

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Hepatocyte Culture on Biodegradable Polymeric Substrates

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Received May 9, 1990/Accepted December 5, 1990

The interactions of primary rat liver cells with biodegradable polymeric substrates were investigated in vitro to assess the suitability of the polymer materials for use in cell transplantation devices. The kinetics of cell adhesion to, and the growth and biochemical function of cells maintained on, films formed from poly (D,L-lactic-co-glycolic acid, 88:12) (PLGA) or from a 50/50 (w/w) blend of PLGA and poly (L-lactic acid) (PLLA) were evaluated in comparison to two control substrates, matrigel coated or collagen-coated polystyrene petri dishes. The rate of cell adhesion to both types of polymeric substrates was similar to the rate of adhesion to the collagen control substrate, but of the two polymers, only the blend was suitable for extended culture. Hepatocytes maintained on the polymer blend films showed retention of differentiated cell function as measured by the rate of albumin secretion—the rate of albumin secretion by cells on the films was the same as the rate for cells on matrigel and reached a level in the range of reported in vivo levels (140–160 $\mu g/10^6$ cells/24 h). In contrast, albumin secretion by hepatocytes maintained on collagen-coated polystyrene culture dishes declined over five days to a level one third that of the initial level and one fifth that of cells maintained on the polymer blend films on day five. Such retention of differentiated cell function by hepatocytes in culture has previously been observed only when hepatocytes were cultured in the presence of exogenous extracellular matrix proteins or were cocultured with another cell type. In addition to retention of differentiated function, the cells maintained on the polymer blend films also displayed rates of DNA synthesis similar to controls maintained on collagen-coated polystyrene, a substrate optimal for DNA synthesis.
Key words: biodegradable polymeric substrates • cell adhesion • liver cell culture • albumin secretion

INTRODUCTION

Polymeric devices are used for replacement of many types of diseased tissues and organs in humans (e.g., artificial joints, breast reconstruction, large diameter vascular grafts, and controlled release of drugs). For these applications, inert surfaces—those which do not interact with proteins or cells—have been used to minimize long term tissue reactions. Most studies of the interactions of cells and proteins with medical polymers have thus been directed toward understanding how to prevent those interactions from occurring. At the opposite extreme is the use of medical polymers as substrates for cell transplantation, in which a high degree of cell–polymer interaction is desired. In this case, the ultimate replacement of the organ or tissue function depends primarily on the transplanted cells; the polymer plays an ancillary role as a substrate for adhesion of the cells and to give structure to the nascent tissue or organ.

Although cell transplantation has been proposed for several cell and tissue types, it is particularly attractive for replacing whole organ liver transplantation[7,11,38] because cell transplantation allows the use of living donors, such as close relatives of the patient, and thus the donor pool would be greatly expanded. A proposed strategy is for cells to be harvested from an appropriate donor, cultured in vitro on a three-dimensional scaffold for 3–5 days for validation of cellular viability, and transplanted into the host where vascularization (and possibly growth) of the cell mass occurs.[38] One of the factors to be resolved before cell transplantation can be applied clinically is the choice of the material for the scaffold. The material should be biocompatible in the sense that it does not provoke a connective tissue response which may impair the function of the new liver tissue, capable of being fabricated into porous three-dimensional devices with a high surface area/volume ratio to provide a significant surface area for cell attachment, and the resulting devices must have sufficient strength to prevent collapse upon implantation. It should be an adhesive substrate for the transplanted cells, and should ideally allow for retention of differentiated function by the cells and possibly for cell growth.

Transplant scaffolds constructed from natural polymers such as collagen, although adhesive for liver cells, have poor mechanical stability, can provoke adverse tissue and immune responses,[28] and may promote undesired cell behavior (e.g., collagen is present in abnormally high amounts in fibrotic liver[9]). In contrast, synthetic polymers can be manufactured reproducibly and have good mechanical properties. Among synthetic materials, resorbable polymers are preferable because permanent materials carry the risk of infection and unfavorable connective tissue response.[16] From the perspective of biocompatibility, biodegradability, and processability, polyesters in the polylactic acid (PLA), polyglycolic acid (PGA), and polylactic-co-glycolic acid (PLGA) family have many desirable features.[15,23,27,30,35]

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Biotechnology and Bioengineering, Vol. 38, Pp. 145–158 (1991)
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CCC 0006-3592/91/020145–14$04.00

These polymers are currently used in humans for surgical sutures and controlled drug release devices. They degrade by hydrolysis and the degradation time can be varied from weeks to over a year by varying the ratio of monomers and the processing conditions.[15,23,27,30,35] Little is known, however, about the interactions of these polymers with cells, especially in ways important for cell transplantation. Characterization of the cell–polymer interactions in vitro is an important first step in predicting the suitability of the polymers for cell transplantation. Furthermore, maintaining appropriate cell-polymer interactions in vitro during short term (3–5 days) culture is essential in the strategy of cell transplantation because demonstration of cellular integrity, which can be irreparably damaged during the cell isolation procedure, must be conducted in vitro before the cells can be implanted.

The interaction parameters of interest are cell adhesion, longevity, and maintenance of differentiated function. Adhesion is desired because the hepatocytes are anchorage-dependent and because it is unlikely that nonadherent cells will stay localized to the site of the implant. It is also desired that cells remain attached and viable on the substrate; since the ideal polymers are erodible, it is conceivable that the attachment sites could degrade during the time span attachment is desired. Finally, for highly differentiated cells such as hepatocytes, the polymer's influence on retention of differentiated function is important to understand. In general, the culture substratum is permissive for the action of hormones and growth factors.[31] Thus, even though different humoral factors may be present in vivo compared to the in vitro culture medium, a substratum that allows retention of function in vitro would be expected to be the optimal substratum for functional retention in vivo.

We have investigated the interactions of hepatocytes with formulations of polymers in the PLA–PLGA family representing a range of physical properties, and report here results with respect to hepatocyte adhesion, cellular longevity, DNA synthesis, and retention of hepatospecific function.

MATERIALS AND METHODS

Cell Culture Substrates

Polymer substrates were made in the form of solvent cast films, and films of two different compositions were investigated. One set of films was made from poly(DL-lactide-co-glycolide) with monomers in the nominal ratio 85:15 lactide:glycolide (DuPont, "Medisorb 85:15"; the lot used for these studies was characterized by DuPont as having an actual monomer ratio of 88:12, average molecular weight (M_w) of 6.7×10^4; M_w/M_n = 1.96; referred to as "PLGA" throughout the remainder of the text) and another set was made from a blend [50/50 (w/w)] of PLGA 85:15 and poly(L-lactide) (Polysciences, $M_w = 5 \times 10^4$). Films were cast in 50-mm-diameter glass petri dishes from a freshly made 15% polymer solution in methylene chloride (Mallinkrodt, analytical reagent grade); each film contained 0.4 g polymer. The dishes containing the films were either covered with petri dish covers and the solvent evaporated at room temperature for a minimum of 5 h, or were left uncovered and the solvent evaporated for 2 h. For films of pure PLGA, the film morphology obtained by either method was smooth and transparent. Thermal transitions of the films were determined using a Perkin Elmer differential scanning calorimeter (DSC 7) using a 10°C/min heating rate. The PLGA polymer is amorphous and showed a single glass transition temperature (T_g) of 53–55°C (variation with lot number) while pure PLLA exhibited a melting temperature of 173–178°C (variation with lot number) with no T_g. The blend films exhibited partial crystallinity and had a T_g of 45–47°C and a melting point of 174–177°C. For films made from the polymer blend, the morphology was highly dependent on the solvent evaporation rate and the lot of PLGA used. For slow evaporation rates, the surface was characterized by numerous small pits 1–5 μm in diameter, while for fast evaporation, the films displayed no pits but were characterized by smooth bumps. Lots of PLGA 85:15 containing less than 85% lactic acid monomer showed phase separation characterized by troughs 50–75 μm deep in the films, and these films were not used. Following solvent evaporation, the films were then placed under vacuum for 24–48 h to remove residual solvent. The films were exposed to UV light for 90 min for purposes of sterilization and were stored dessicated until use. The films were prepared for culture by washing once with 5 mL phosphate buffered saline (PBS, pH 7.4) and then once with 5 mL complete culture medium (described below). Control petri dishes (35 mm bacteriologic, Falcon No. 1008) were coated with Type I collagen (Vitrogen, Collagen Corp.) by adsorption from a 5 μg/mL solution of Vitrogen (2 mL/dish) in 50 mM carbonate buffer (pH 9.4) for 16–20 h at 4°C; the resulting surface concentration of collagen was 1 μg/cm^2. Coating of polymer films with Vitrogen was achieved by adsorption from a 50 μg/mL solution in PBS, pH 7.4 (5 mL/dish), for 6 h at room temperature. For culture on basement membrane matrigel, 200 μL ice-cold matrigel (Collaborative Research) was spread on the bottom of each 35 mm dish using a cell scraper and allowed to gel at 37°C for 60 min before use.

Cell Isolation and Culture

Cells were isolated from 180–250 g male Fisher rats using a modification of the two-step collagenase procedure of Seglen.[34] The liver was perfused in the retrograde direction first with Ca^{2+}-free perfusion buffer (143 mM NaCl, 7 mM KCl, 10 mM HEPES, pH 7.4) for 6 min and then with the same perfusion buffer contain-

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ing 5 mM $CaCl_2$ and 0.5 mg/mL collagenase (Worthington, Class II) until the liver became soft. Cells were dispersed in chemically defined serum-free culture medium [William's E with 10 ng/mL EGF (Collaborative Research), 20 mU/mL insulin (Gibco), 5 nM dexamethasone (Sigma), 20 mM pyruvate (Gibco), and 100 U/mL penicillin/streptomycin (Gibco)].[26] This medium will subsequently be referred to as "complete medium." Cell viability following dispersion was 80–90% as determined by trypan blue exclusion. Dead cells and debris were removed by centrifugation in an isodensity Percoll solution[22] and the resulting pellet was then washed three times with complete medium. Viability at plating was 88–98%.

For routine culture, cells were plated in complete medium at a concentration of 3×10^4 viable cells/cm^2 culture surface area (3×10^5 cells/dish for 35-mm control dishes, 6×10^5 cells dish for 50-mm polymer films; 1.5×10^5 cells/mL). Following an attachment period of 2–4 h (maximum attachment to all substrates occurred within 90 min), the medium was changed to remove unattached cells and then cells were maintained in complete medium with daily medium changes.

Cell Attachment

Cell attachment to substrates was measured by direct counting or by determining relative protein content after the cell layer had been washed two times with PBS; in both cases, replicate plates were sacrificed for each measurement (cells enumerated by direct counting were not replated). For direct counting, cells were removed from the polymer films or collagen-coated polystyrene using 0.05% trypsin/EDTA (Gibco). Cells plated on collagen-coated substrates required prolonged (30–45 min) treatment with trypsin for adequate cell dispersion, and plates were inspected visually before counting to ensure all cells had been removed from the substrate. Cell removal from matrigel using Dispase digestion as recommended in the matrigel package insert was found to be incomplete, and a method based on incubation in an EGTA solution was used to obtain complete removal. For removal with EGTA, cultures in 35-mm dishes were washed twice with 2 mL 0.5 mM EGTA in PBS (pH 7.4)[24] and incubated for 16 h in 2-mL EGTA solution. Following the incubation, the matrigel could be disruputed by gentle tituration, and the cells were recovered intact.

For determining cell number on a large number of polymer films or collagen-coated plates simultaneously, quantitative binding and extraction of the dye flavianic acid (NYS, Sigma) was used.[36] Briefly, cells were fixed in cold 10% TCA for 30 min followed by staining with 0.2% flavianic acid in 1% acetic acid for 30 min. The staining solution was then removed, the cultures rinsed with 1% acetic acid, and the excess unbound dye was removed by four successive incubations of 2.5 min each with 1% acetic acid (2 mL each rinse for 35-mm plates, 4 mL each rinse for 50-mm dishes). Plates were air dried, and the bound dye was extracted with unbuffered 10 mM Tris base (2 mL for 35-mm dishes, 4 mL for 50 mm dishes) for 5 min. Extracted dye was diluted 1:2 with 10 mM Tris base and the absorbance of each sample was read at 433 nm relative to 10 mM Tris. To calibrate the assay for hepatocyte cell number, replicate plates were seeded at cell concentrations of 0, 3×10^5, and 6×10^5 cells/plate. At each cell concentration, three plates were counted and three plates were used for the NYS assay. A reproducible linear response of absorbance vs. cell number was obtained; with data from three separate experiments, the linear correlation was $A_{433} = -0.005 + 1.8\text{MC}$ ($R = 0.961$), where MC is the cell number in millions. Blank polymer films (both at time zero and after incubation in culture medium for five days) and collagen-coated plates registered absorbance values of 0.004 or less.

The rate of cell attachment was measured by seeding replicate plates at 3×10^4 cells/cm^2 surface area. At each time point, three identical plates were sacrificed for measurement of cell number by the NYS dye-binding assay.

For determining effects of the presence of extracellular DNA from dead cells on cell attachment at high cell concentrations (5×10^5 cells/cm^2), deoxyribonuclease I (United States Biochemical Corp.) was added to the attachment medium at concentrations 0, 10, 100, and 1000 U/mL. Cells were plated on the standard Vitrogen-coated, 35-mm polystyrene dishes and attachment was determined on duplicate plates.

For determining the effects of albumin on cell attachment, cells were seeded at 3×10^4 cells/cm^2 in 5 mL complete medium containing 20 μg/mL albumin (bovine fraction V, cell culture tested, Sigma). The albumin-containing medium was added to the substrate at the same time as the hepatocytes. The hepatocytes in this study typically secrete albumin at approximately 100 μg/million cells/day, and at the cell concentration used in the attachment experiment, the expected accumulation of albumin in the medium after 24 h would be 7.5 μg/mL.

Analytical Assays

Albumin secretion into the culture medium was quantified by a sandwich ELISA technique[33] using antibodies specific for rat albumin (Organon-Teknika-Capell). DNA synthesis was measured biochemically by the incorporation of 1 μCi/mL ^{3}H-thymidine over 20 h. Total DNA content and radioactivity were determined according to the method of Bucher.[22] For determining the specific DNA synthesis rates on matrigel, the DNA content was corrected by subtraction of the DNA content of matrigel-coated dishes without cells.

Electron Microscopy

Cell cultures were fixed in Karnovsky's fixative at 37°C for 1 h, washed with 0.1M cacodylate buffer (pH 7.4),

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postfixed for 1 h in 1% osmium tetroxide, dehydrated in a graded series of ethanol–water solutions, and dried in a critical point drier (Ladd) with supercritical CO_2. The samples were then sputter-coated with gold and observed in a Hitachi scanning electron microscope.

RESULTS

Cell Attachment

In one set of experiments, the kinetics of cell attachment to substrates were studied at cell surface concentrations of 3×10^4 cells/cm^2 and below to minimize the role of cell-cell interactions. There are two relevant cell concentrations: a two-dimensional surface concentration and a volumetric concentration. The two-dimensional concentration is relevant because the cells settle to the surface quickly regardless of the total volume, and the surface concentration reflects the degree to which cells may interact at the attachment interface and the relative competition for sites; i.e., for cells 20–25 μm in diameter, total surface coverage with spheres would correspond to $(1.6–2.5) \times 10^5$ cells/cm^2, so 3×10^4 cells/cm^2 would represent less than 20% surface coverage. The volume concentration is important because attachment may be inhibited by cell-secreted proteins.[14] The rate of hepatocyte attachment to the two types of polymer films and to control collagen-coated petri dishes is shown in Figure 1. The rate of attachment to both types of polymer films is similar and maximum attachment is achieved in 60 min, while maximum attachment to the collagen-coated control dishes required about 120 min. The kinetics of attachment to collagen are similar to other reported rates.[20] Hepatocytes were observed to attach primarily as single cells, and no difference in the kinetics of attachment to collagen-coated dishes was observed at a fourfold lower cell concentration (7500 cells/cm^2, data not shown).

At cell concentrations above 10^5 cells/cm^2, cell–cell interactions significantly affect the number of cells attaching to the substrate and the pattern of hepatocyte attachment is quite different for the polymer substrates and controls. On all substrates, the number of cells attached to the substrate as a function of the number of cells plated showed a maximum (Fig. 2). The maximum number of cells which attached to uncoated polymer substrates was 5×10^4 cells/cm^2, while cell attachment to collagen-coated polymers or petri dishes was significantly higher $(8.0–9.5) \times 10^4$ cells/cm^2. At high cell concentrations, the cells formed aggregates of 2–10 cells, and while these aggregates could be seen to attach to the collagen-coated substrates, they did not appear to interact with the untreated polymer films. The formation of cell aggregates was not affected by the presence of DNAase (100–1000 U/mL), as would be expected if the leakage of DNA from dead cells was causing nonspecific aggregation.

Even though a much greater number of cells attached to the collagen-coated substrates than to the polymer films at high cell concentrations, cell-cell interactions also influenced the pattern of attachment at concentrations of cells above 2×10^5/cm^2; during the washing step, patches of cells would come up from the surface, leaving the surface bare in spots. The distribution of cells on the surface can be easily visualized using the NYS assay, and such patchiness was not observed at cell concentrations of 2×10^5 cells/cm^2 and lower. At 10^6 cells/cm^2, cell attachment was inhibited on all the substrates and cell–cell aggregation predominated.

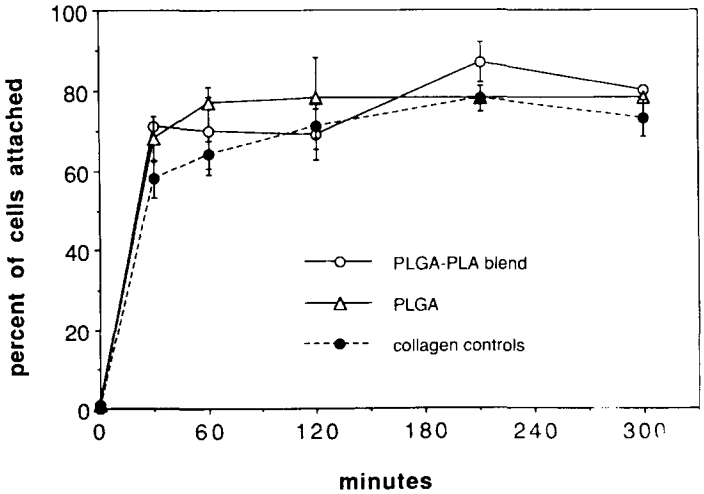


Figure 1. Rate of cell attachment to polymer films and control collagen-coated polystyrene petri dishes for cells seeded at 3×10^4 cells/cm^2. Each point represents the average of three plates for collagen controls and two plates for polymer films (mean ± standard deviation).

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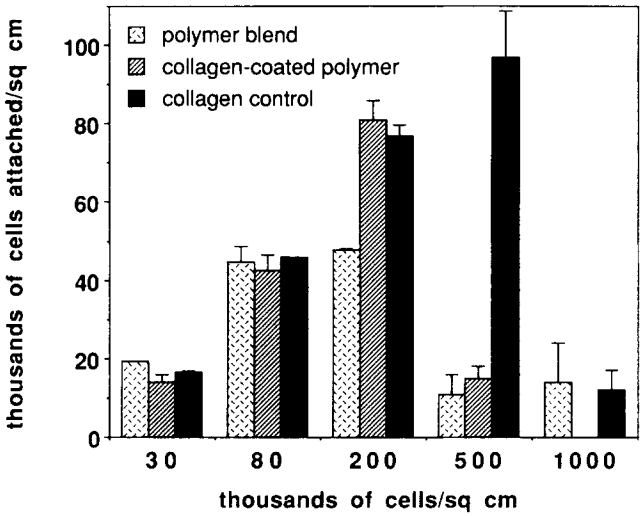


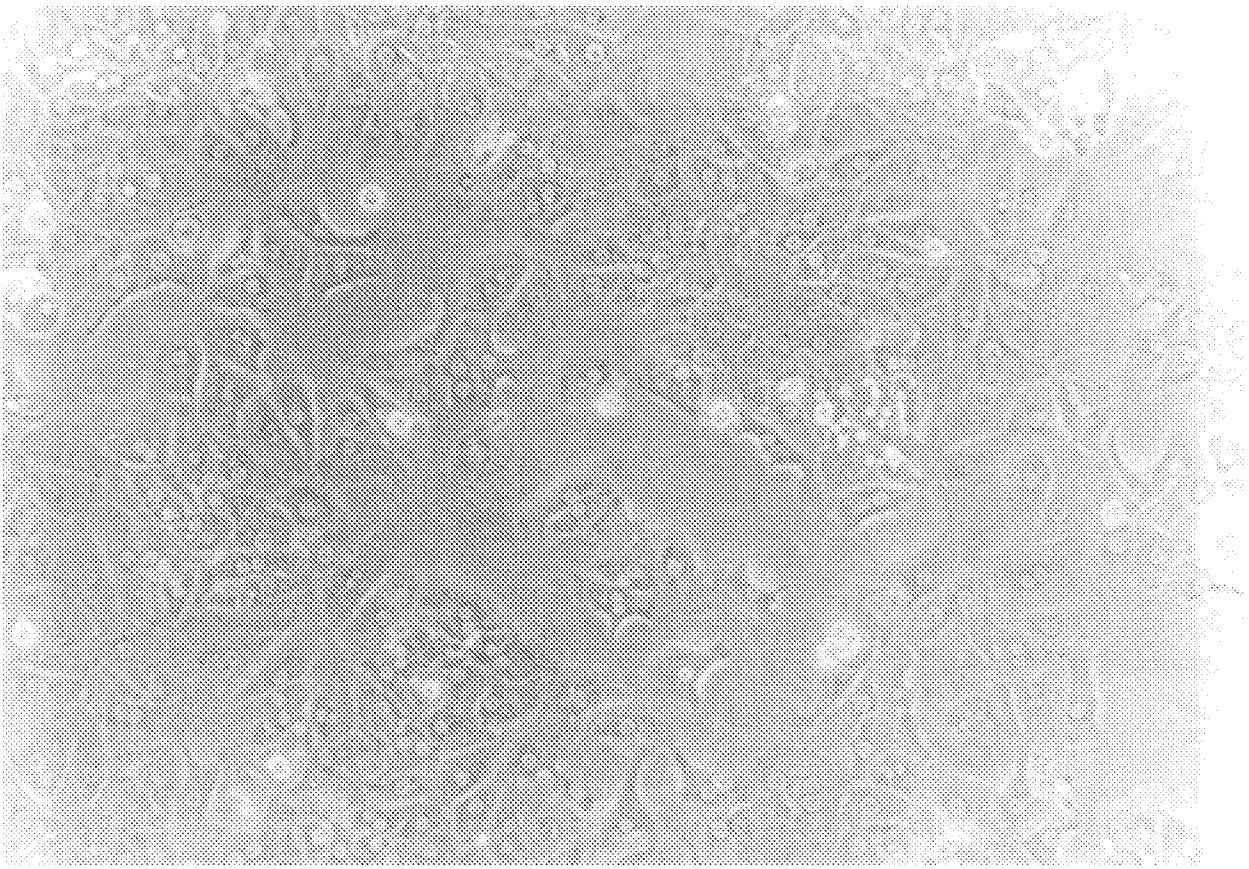
Figure 2. Number of cells attached as a function of the number of cells available at the attachment interface per cm^2. Each point represents the average of two plates (mean ± standard deviation). (Attachment was not measured for collagen-coated polymers at 10^5 cells/cm^2.)

Cell attachment to the polymer blend substrate at a low cell concentration (3×10^4 cells/cm^2) was not affected by the presence of albumin (20 μg/mL) in the attachment medium

Cell Morphology

Scanning electron microscopy (SEM) was used to observe the morphology of the cells on the polymer substrates due to the opacity of these substrates and a comparison between the morphology obtained by SEM and standard phase contrast light microscopy is shown in Figures 3(a) and 3(b). One day after plating, the cells maintained on the collagen-coated polystyrene dishes were highly spread and flat [Figs. 3(a)–3(c)]; surface microvilli were observed predominantly in the center of the cells [Fig. 3(c)]. In contrast, the morphology of cells on the polymer substrates was heterogeneous; both highly rounded and spread cells were observed [Figs. 3(d) and 3(e)]. On the polymer films, both rounded and spread cells had numerous surface microvilli [Fig. 3(e)]. The strands of extracellular matrix apparent in the SEM micrographs were not characterized chemically in this study, but appear similar morphologically to the fibronectin and fibrin strands which have been observed to be elaborated by hepatocytes in serum-free medium.[37] Cells maintained on matrigel displayed a highly rounded cell configuration [Fig. 3(f)].

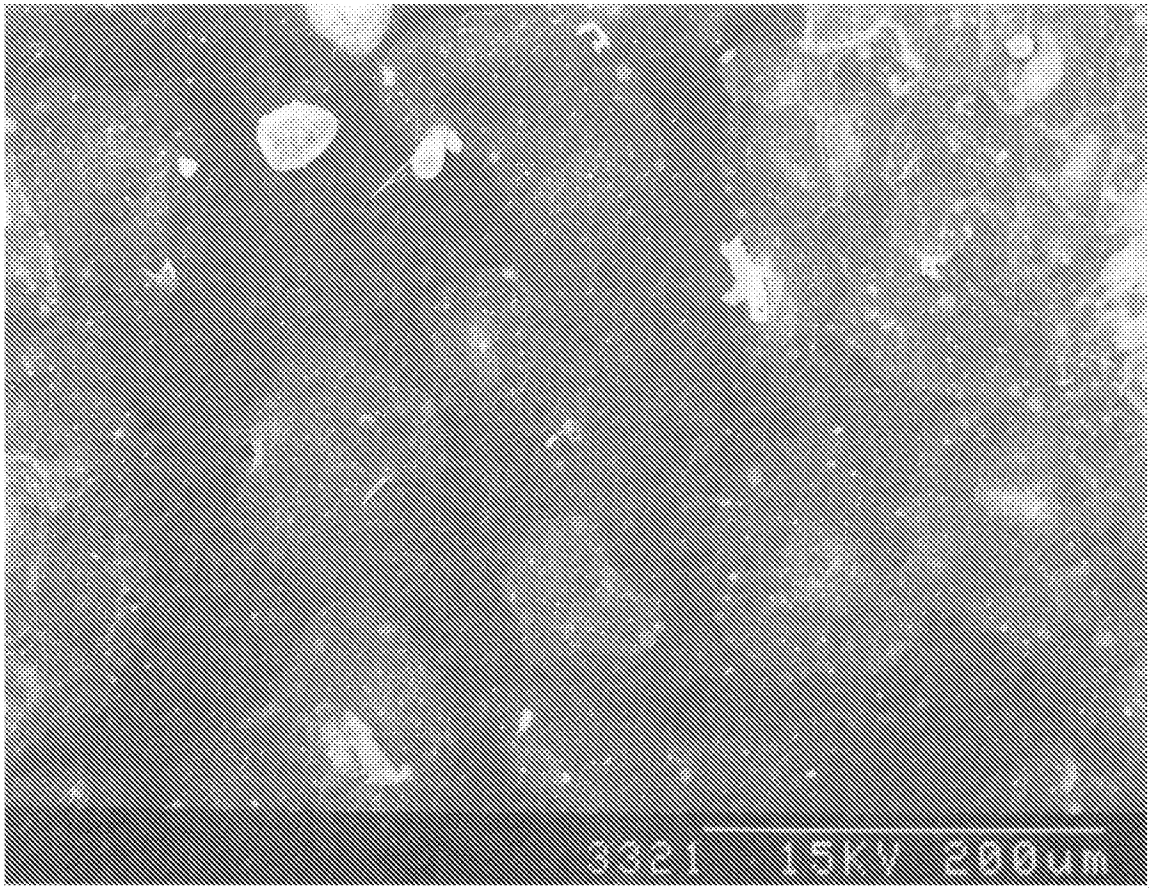
After four days in culture, cells on collagen remained spread [Fig. 3(g)]. On the poymer blend films, very few individual cells remained isolated and rounded; most



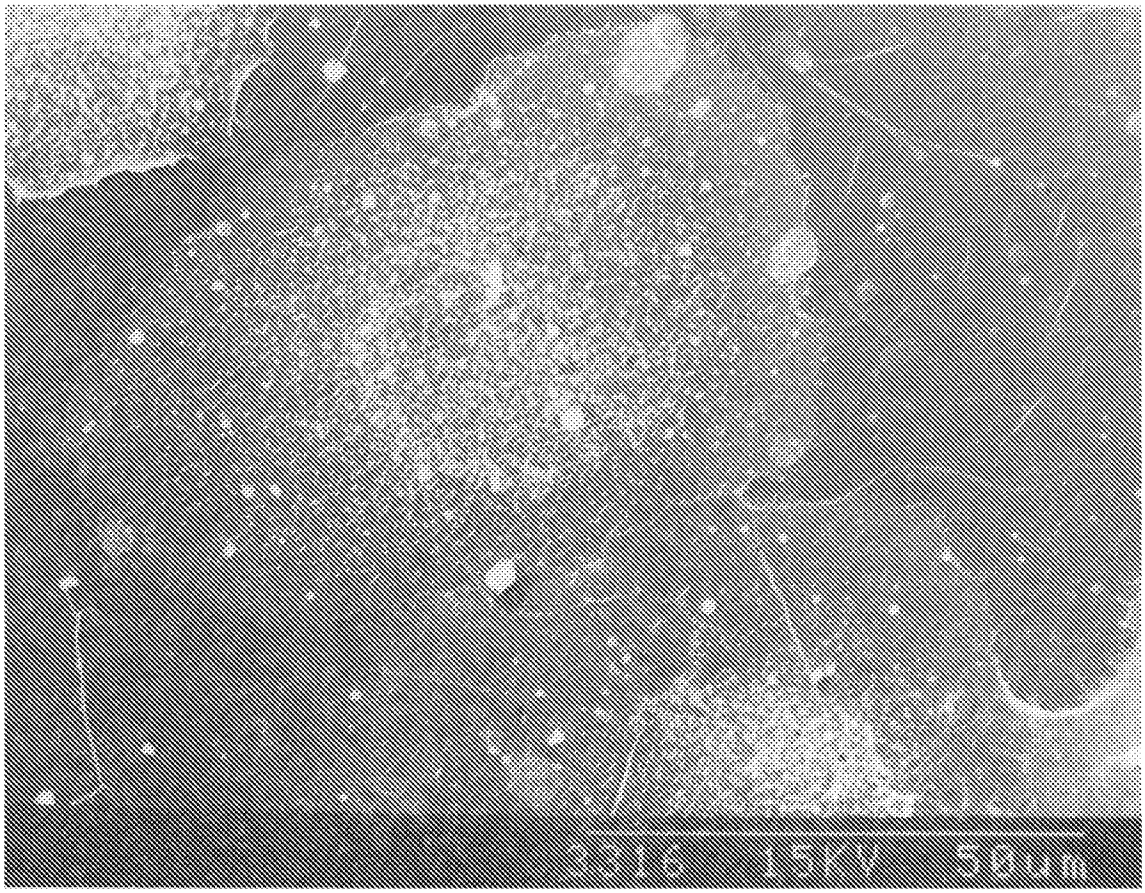
(a)

Figure 3. Cell morphology on collagen controls, polymer blend films, and matrigel after 18 h (a–f) and four days (g–i) of culture. The initial cell surface concentration for all substrates was 1.5×10^4 cells/cm^2. Phase-contrast light micrographs (original magnification 40×) are of cells on collagen (a, g) and matrigel (f, i). Scanning electron micrographs are of cells on collagen (b, c) and polymer blend [50/50 (w/w) 85:15/PLLA] film (d, e, h).

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(b)

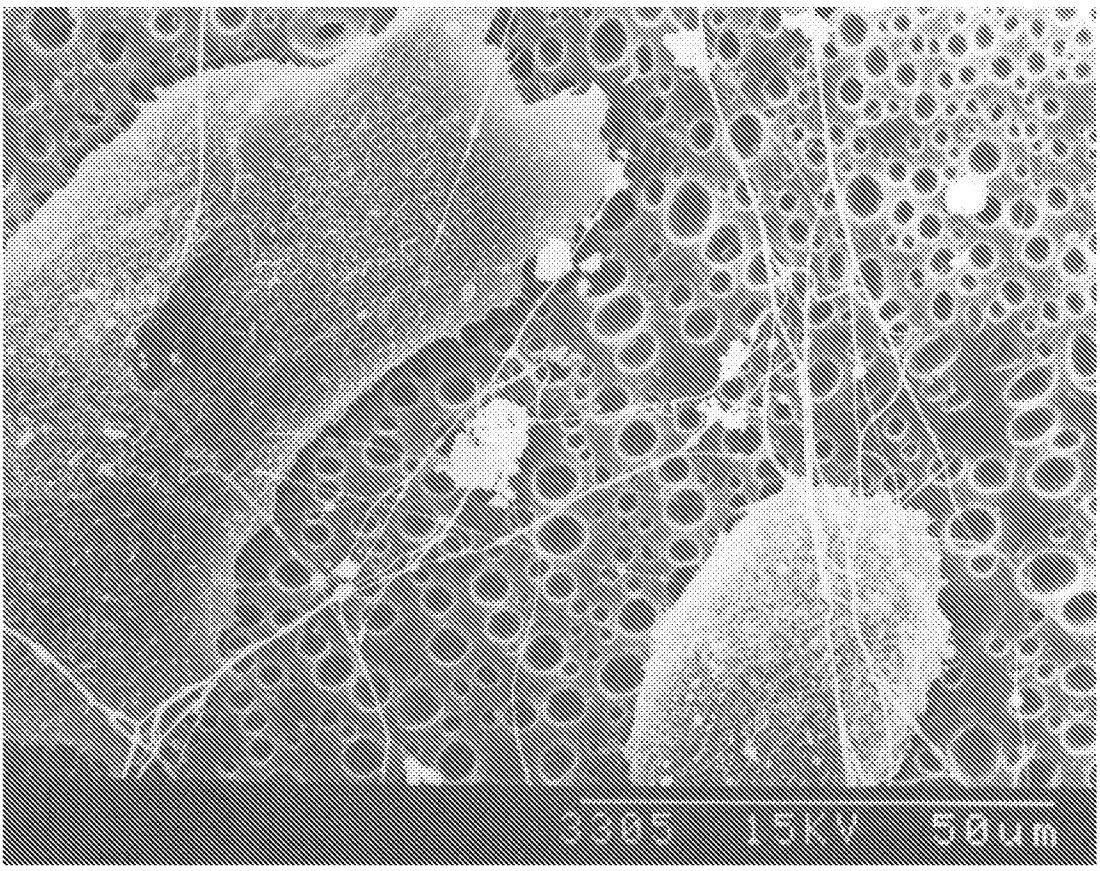


(c)

Figure 3. (*continued*)

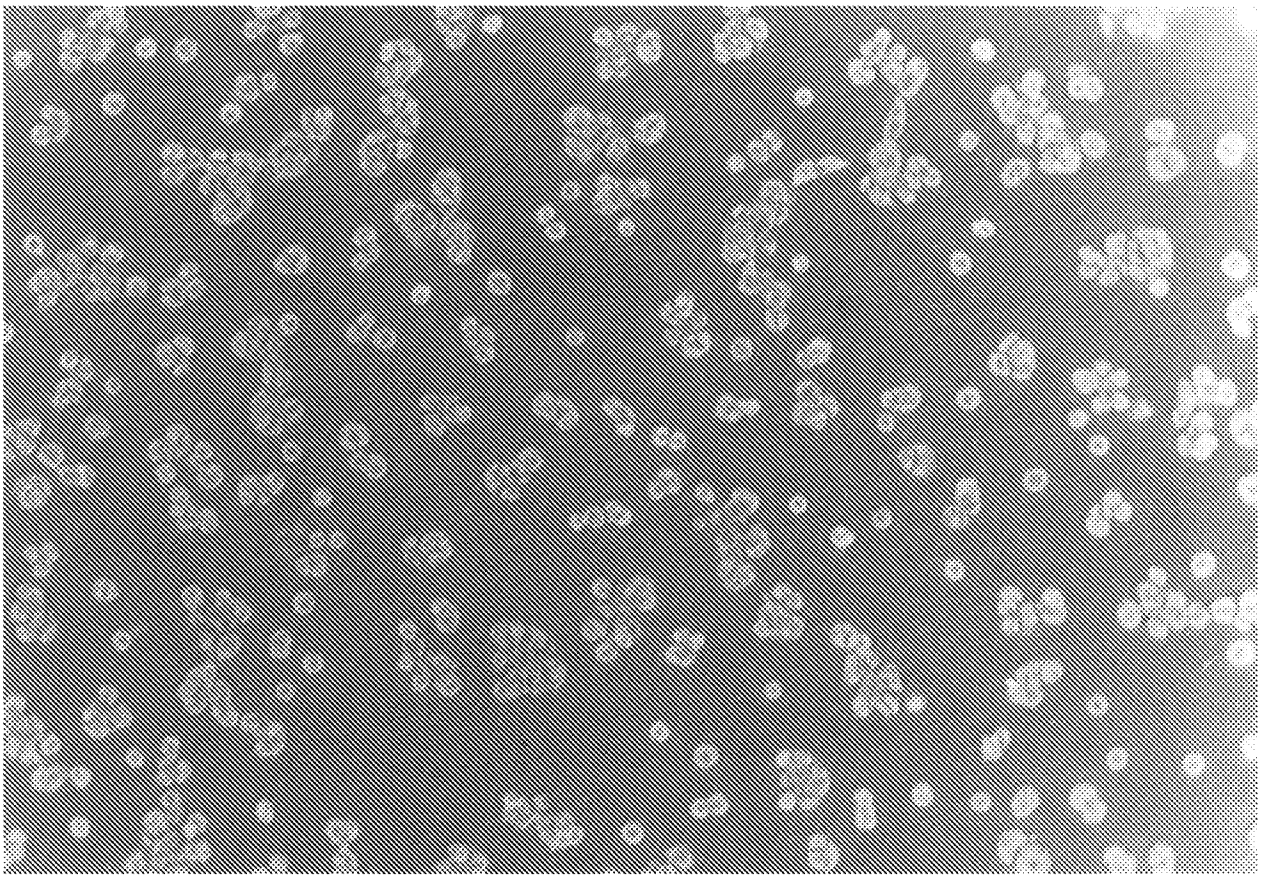


(d)

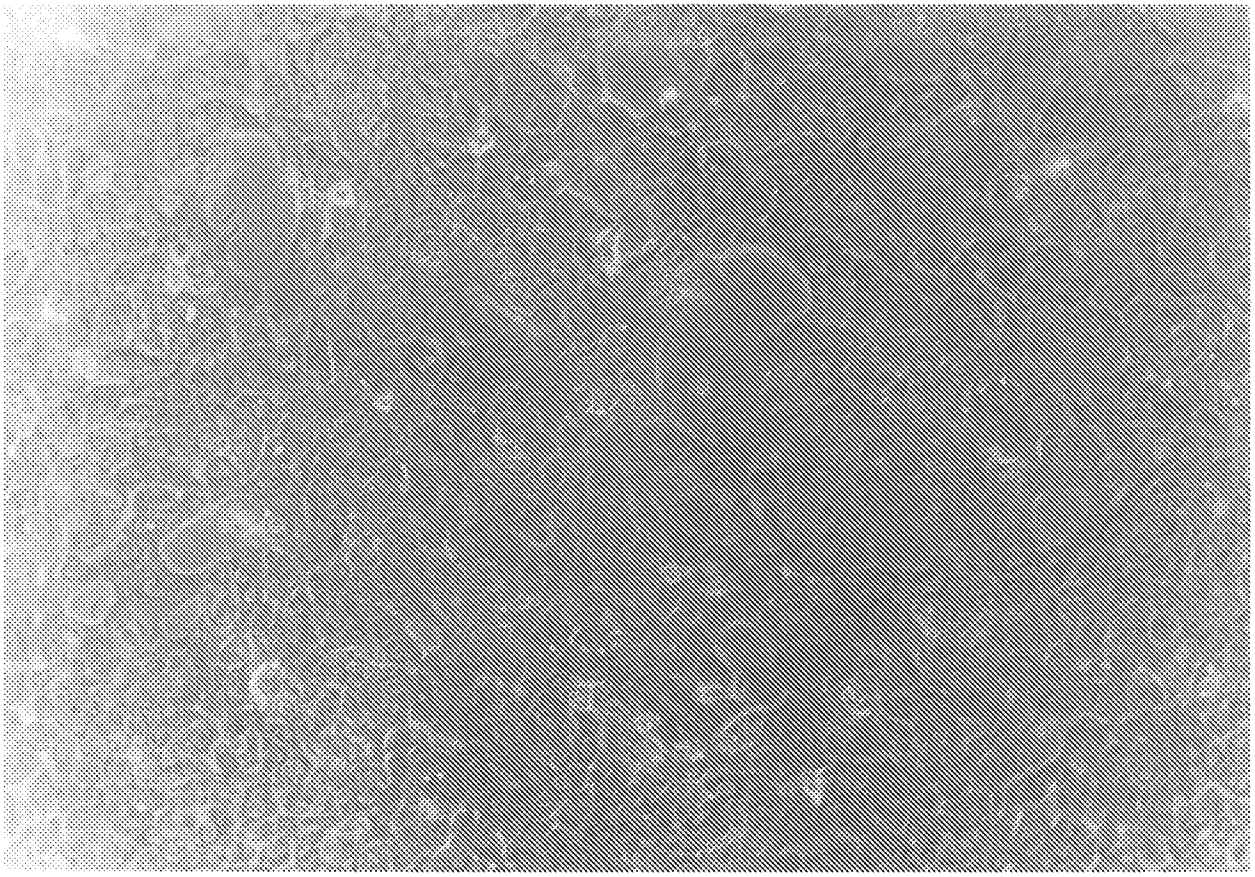


(e)

Figure 3. (*continued*)

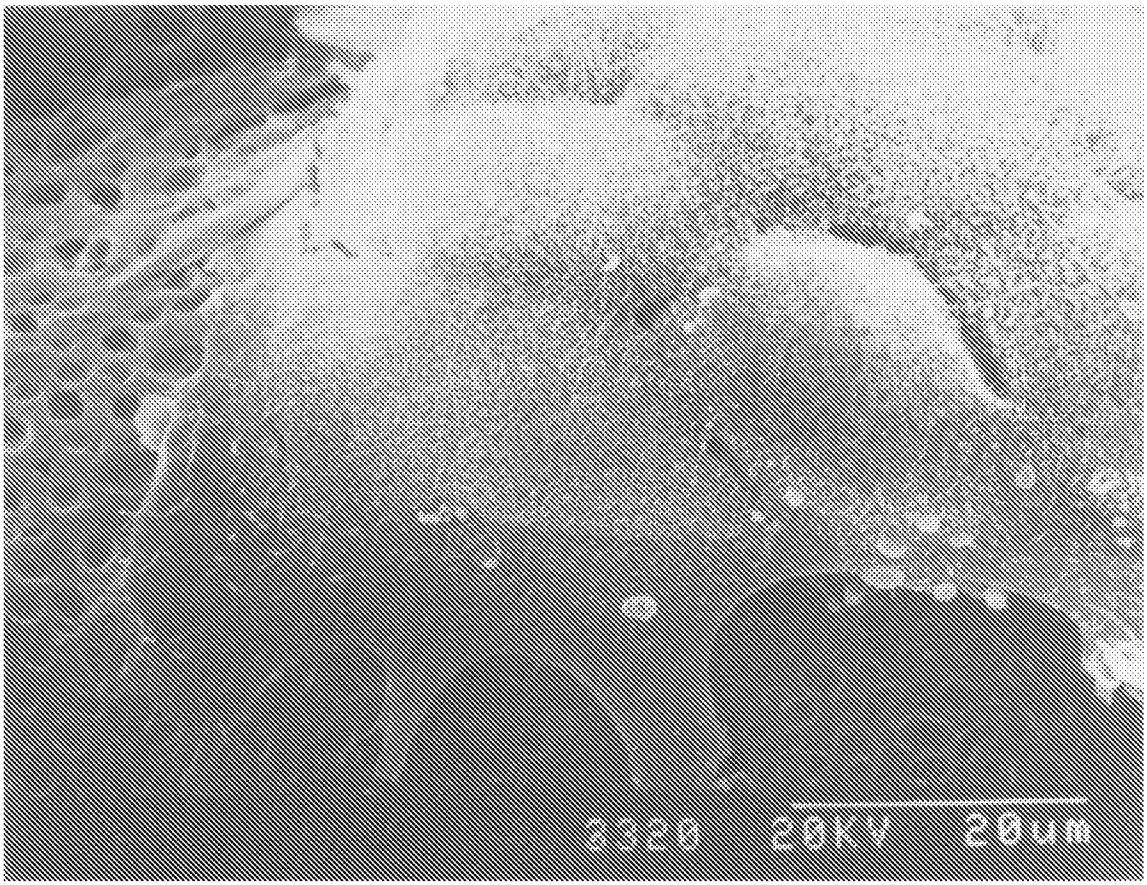


(f)

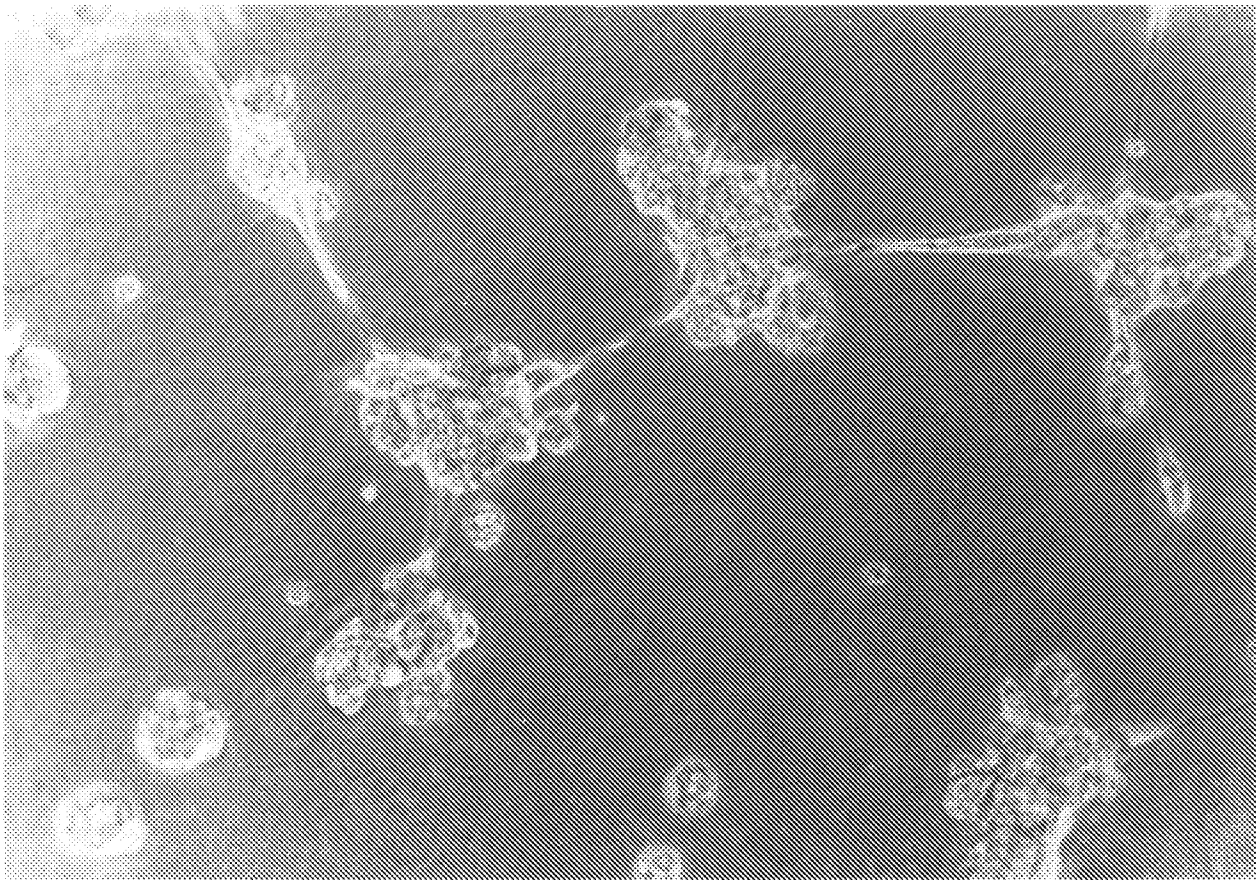


(g)

Figure 3. (*continued*)



(h)



(i)

Figure 3. (*continued*)

cells associated into large groups [Fig. 3(h)]. In contrast to the behavior of cells on collagen, where cells at the periphery of a cell sheet were observed to be highly flattened and spread, cells at the periphery of cell groups on polymer blend films were mostly rounded, although some flattening and extension of the cells was occasionally observed [Fig. 3(h)]. Cells on matrigel aggregated into trabecular structures [Fig. 3(i)] of similar morphology to those reported elsewhere.[4]

The surface morphology of the polymer blend films was rough, with pits up to 2 μm in diameter [Figs. 3(a) and 3(c)]. The surface morphology of these films appeared unchanged over time in culture, and the same morphology was observed in the original, dry state. The macroscopic appearance of the blend films, however, changed from an initial transluscence to opaque white after 12 h in contact with culture medium. In contrast, the films made from pure PLGA were initially smooth and transparent, and remained so during the cell culture experiments.

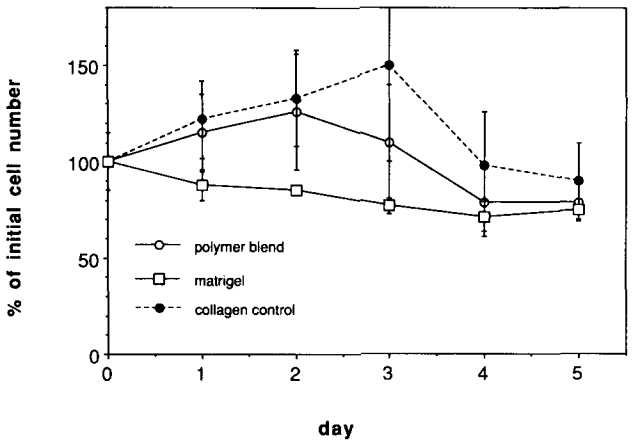


Figure 4. Cell longevity on polymer blend films and control collagen-coated polystyrene dishes. The experiment was done twice using duplicate plates for both conditions at each time point; each point shown represents the average of four plates, (mean ± standard deviation). The cell surface concentration for both substrates initially was in the range of (1.2–1.5) × 10^4 cells/cm^2. (Cell surface concentrations were similar on both substrates for a given experiment, but were slightly different for the two different experiments.)

Cell Growth and DNA Synthesis

The growth pattern of hepatocytes maintained on polymer films cast from the blend of PLA and PLGA is similar to that of hepatocytes maintained on the collagen control substrates (Fig. 4) and the rate of incorporation of ^{3}H-thymidine into DNA are equivalent (Fig. 5). Attrition of cells begins after three days in culture. Such attrition has been reported in many hepatocyte culture systems.[12,20,32] The behavior of cells on the collagen and polymer blend films is in contrast to the behavior of hepatocytes on matrigel, where the number of cells gradually declines and the rate of incorporation of ^{3}H-thymidine into DNA is about 25% that of the cells on collagen.

The films cast from pure PLGA were not good substrates for cell longevity at the cell concentration investigated (2 × 10^4 cells/cm^2). After 3–4 days, the cells formed cordlike structures and began to detach from the substrate in large wisps. We have observed similar detachment of hepatocytes from the substrate when cells are cultured on fibronectin-coated polystyrene dishes

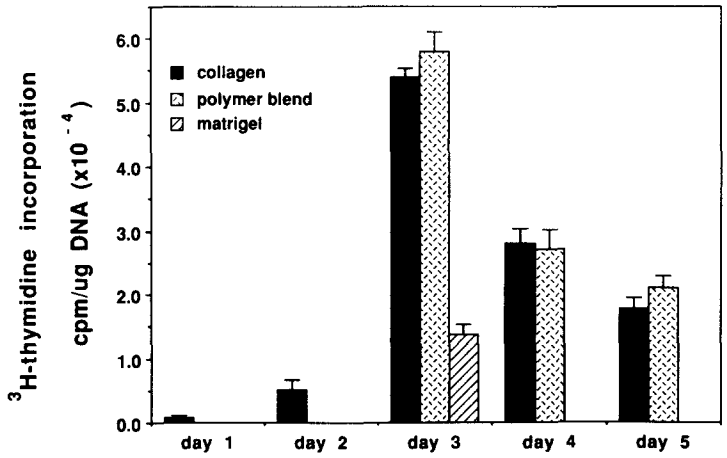


Figure 5. Rate of incorporation of ^{3}H-thymidine into DNA during a 20-h labelling period for cells on polymer blend films and control collagen-coated polystyrene petri dishes. The time corresponding to each point is the end of the labelling period and represents the average of at least two experiments (mean ± standard deviation). It was determined for cells on collagen controls that negligible DNA synthesis occurred before day 3, so rates for polymers were measured only days 3–5 and for matrigel only day 3. (Cell surface concentrations were similar on both substrates for a given experiment, but were slightly different for the two different experiments.)

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(10 $\mu g/cm^2$; (1–3) $\times$ 10^4 cells/cm^2). Such detachment of cell sheets or cords from the substratum has also been observed in other systems and has been attributed to cell-cell tensions that overcome the cell-substratum tension.[18]

Functional Behavior of Hepatocytes

The retention of hepatocellular function on polymer blend films was assessed by measuring the rate of albumin secretion. The rate of albumin secretion ($\mu g/10^6$ cells/day) by cells maintained on the polymer blend films increased almost twofold over the five days in culture (Fig. 6); in contrast, albumin secretion for cells on the collagen control dishes decreased over 60% (Fig. 6) in accordance with trends previously reported.[4,8,32] The decline in albumin synthesis for cells maintained on collagen was the same for collagen surface concentrations of 1 and 10 $\mu g/cm^2$. The secretion rate of albumin by cells on the polymer blend films is similar to cells maintained on matrigel (Fig. 6), comparable to that of cells maintained in collagen-gel sandwiches[12] (100–135 $\mu g/10^6$ cells/day) and is in the range of the reported in vivo rate for rats (17.3–19.4 mg/g liver/24 h[29] which corresponds to 144–162 $\mu g/10^6$ cells/24 h based on 120 $\times$ 10^6 hepatocytes/gm liver[39]). The initial rate of albumin secretion for cells maintained on collagen (88 $\pm$30 $\mu g/10^6$ cells/day) we observed is close to that reported by Sheutz et al. for cells maintained on Vitrogen gel (73.4 $\pm$10.4 $\mu g/10^6$ cells/day).[32]

DISCUSSION

The first parameter defining a good substrate for cell transplantation is cellular adhesion to the substrate. Prediction of cellular adhesion to synthetic biomaterials is difficult because the interactions of physical and chemical properties of the surface are complex; hydrophobicity, charge, specific chemical groups and degree of water saturation all play important roles[1,25,40] and adhesion cannot be predicted on the basis of one parameter. In addition, proteins such as fibronectin and vitronectin which are present in the serum-supplemented media used in many adhesion studies are known to mediate cell adhesion. These studies were carried out in the complete absence of exogenous serum because addition of exogenous extracellular matrix proteins is undesirable in cell transplantation. Such proteins may be immunogenic or derived from unsafe sources such as tumor tissue. While it may be possible to coat the transplant matrix with serum from the patient, the adhesion proteins present in serum are not necessarily representative of the ECM molecules in the normal environment of the hepatocytes. Although serum fibronectin promotes initial cell adhesion, it is not an optimal substrate for retaining cell differentiation.[37] Also, serum contains other factors which contribute to dedifferentiation of hepatocytes in vitro.[31] Adhesion in these studies is mediated initially by the proteins and proteoglycans on the cell surface, and the degree of spreading on the surface may then be mediated by ECM proteins secreted by the hepatocytes subsequent to the initial adhesion event.

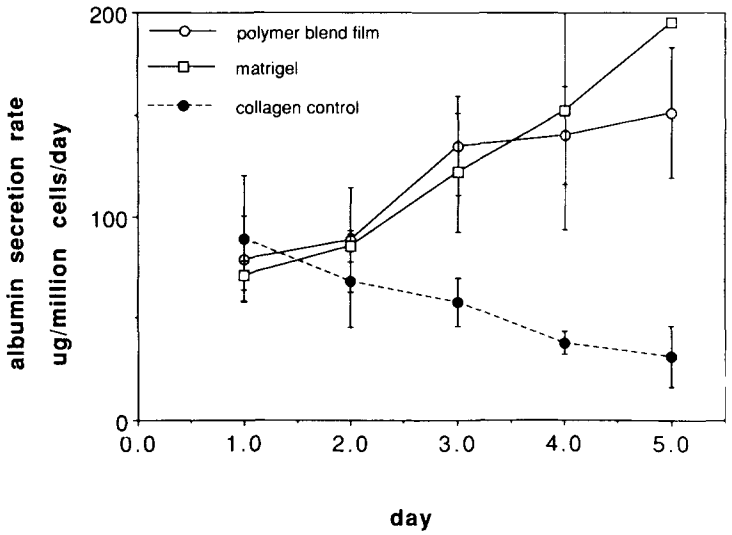


Figure 6. Retention of albumin secretion rates ($\mu g/10^6$ cells/day) for cells maintained on polymer blend films and collagen-coated polystyrene petri dishes. The experiment was done twice with duplicate plates for collagen and matrigel and triplicate plates for polymer films at each time point; each point shown thus represents the average of four (collagen) or six (films) plates (mean $\pm$ standard deviation). (Cell surface concentrations were similar on both substrates for a given experiment, but were slightly different for the two different experiments.)

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The maximum cell concentration that could be accomodated on the polymer surface in serum-free medium with no exogenous ECM proteins added was 5×10^4 cells/cm^2, and the number of cells attached to the substrate declined if the density of cells plated at the surface exceeded 10^5 cells/cm^2 unless the surface was coated with collagen. The attachment of hepatocytes to the polymer films is similar in some respects to the attachment of hepatocytes to tissue–culture polystyrene.[14] Adhesion of hepatocytes to polystyrene is inhibited at high cell concentrations in a cell concentration dependent manner, and this has been attributed to the quenching of non-specific attachment sites by albumin produced by the hepatocytes.[14] However, we found that addition of albumin to the attachment medium—at a concentration more than twice that which would accumulate in the medium over 24 h for the cell number used—did not inhibit cell attachment to the polymer at a cell concentration of 3×10^4 cells/cm^2, where cell attachment to both polymer and the control were at a maximum. Hepatocyte attachment to other synthetic surfaces in the absence of serum or exogenous matrix proteins, using comparable cell concentrations to those used here, has been reported to be significantly lower than attachment to those same substrates in the presence of serum or ECM proteins.[21] In a study comparing attachment of rat hepatocytes to poly(acrylonitrile) ultrafiltration membranes which had either been untreated or etched to expose carboxyl groups, only 15–21% of cells attached when cells were seeded at 10^5 cells/cm^2 compared to 50–60% observed in this study.[21]

The maximum number of cells which attached to the polymer substrate is approximately 30% of that which would be predicted for a completely covered surface based on 25-μm-diameter cells (double layering of cells was observed at cell surface concentrations of 1.9×10^5 cells/cm^2 and above[20]). A cell surface concentration of 5×10^4 cells/cm^2 is a desirable cell density in implant situations where cell growth is desired, and should also be in the effective range if no cell growth is planned. If more efficient use of the polymer surface is required, cell adhesion may be enhanced by coating the polymer substrates with ng-μg/cm^2 amounts of extracellular matrix proteins, provided such coating does not compromise cell functional integrity. Coating the substrate with type I collagen at a nominal concentration of 10 μg/cm^2 increased the maximum cell adhesion to 8×10^4 cells/cm^2. At cell surface concentrations in this range, approximately 625 cm^2 surface area would be needed to replace 5% of the liver mass in a 250-g rat.

In addition to adhesion, other aspects of the cell-polymer interactions may affect the success of the implanted cells in performance as replacement tissue. It has been observed that hepatocytes lose liver-specific functions in culture when maintained on simple substrata such as tissue culture plastic[31] and Type I collagen-coated polystyrene,[4,32] but that high levels of hepatospecific function can be maintained by culturing the cells on complex substrata such as basement membrane extracts.[3,4,32] It has also been reported that albumin secretion rates by cells maintained on simple substrata, after initial decline, can be restored to near in vivo levels by the addition of extracellular matrix proteins.[8] In order to retain hepatospecific function in vivo, we expect that the cells will have to be implanted in a highly functional state, and that best way to ensure this is using a substratum for attachment which is also optimal for allowing retention of cell function. Hepatocytes cultured on films made from a blend of PLGA and PLLA displayed retention of an important hepatocellular function (albumin secretion, Fig. 6) at a level similar to that for basement membrane matrigel. One possible mechanism of the observed retention of differentiated function is through modulation of cell shape; control of gene expression in many cell types has been demonstrated to be cell shape-dependent.[2,3,5,6,19] Regulation of hepatocellular function by cell shape has also been postulated as a mechanism for retention of hepatocellular function by cells maintained on matrigel,[3,4] a substrate on which cells remain extremely rounded, and in high-density culture.[3] Although we have no quantitative data on the degree of cell spreading on the polymer blend films versus the collagen controls, the morphology of the cells on the two different substrates is quite different and the cells qualitatively appear more rounded on the films (Fig. 3). However, the observation by Caron[8] that the decline in albumin secretion by cells maintained on collagen can be reversed and the albumin secretion restored to near in vivo levels by adding exogenous laminin does not support this theory because the addition of the laminin was apparently unaccompanied by any morphological changes by the cells.[8] Another possible mechanism for the increase in albumin secretion rates is that other liver cell types (e.g., lipocytes) present in the initial isolated cell suspension adhere more readily to the polymer films than to the controls and these cells may contribute either extracellular matrix or some type of direct cell–cell interaction which allows functional retention by the cells. High degrees of liver specific function are retained in culture when hepatocytes are cocultured with another liver epithelial cell type,[17] presumably due to ECM and other factors secreted by the nonhepatocyte cell type. However, successful coculture systems employ at least 1:1 ratios of hepatocytes to coculture cell type. Our system had very few contaminating cells as observed by light microscopy of cultures and tryspinized cells (hepatocytes are significantly larger than other hepatic cells), and the rate of albumin secretion on a per cell basis is comparable to that reported elsewhere.[12,32] Thus, the coculture effect is not a likely mechanism for the observed results.

One intriguing observation is that DNA synthesis levels were the same for cells on the polymer blend

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films and the collagen-coated controls (Fig. 5). Hepatocytes have previously been reported to exhibit an inverse relationship between growth and differentiation in vitro,[3] and we thus would expect to find lower levels of DNA synthesis on the polymer films, where the albumin secretion rate was high, than on controls, where albumin secretion was low. This pattern of retention of differentiated function by cells undergoing DNA synthesis on the polymer blend films is similar to the reported behavior of hepatocytes in regenerating liver.[13] Friedman et al. reported that the levels of several liver-specific mRNAs did not change significantly in mouse liver during regeneration following 70% hepatectomy, and that transcription rates of the same mRNAs were generally unchanged or only slightly changed during regeneration. In particular, the transcription rate of albumin mRNA showed a transient decrease starting 4–6 h following hepatectomy, but returned to near-normal levels within 24–48 h; the first wave of DNA synthesis occurred at 36 h.[13] For cells on the polymer blend films, we observed an increase in albumin secretion levels during the first 48–72 h, with values beyond 72 h in the range of in vivo albumin secretion[29,39]; the peak of DNA synthesis in vitro occurs between 48 and 72 h (Fig. 5).

At the outset of this study, it was anticipated that polymer materials suitable for cell transplantation from a biocompatability and mechanical perspective may have to be modified in some manner (e.g., by adsorption of extracellular matrix proteins to the surface) to enhance cell adhesion or retention of differentiated function; retention of function by hepatocytes on simple synthetic substrates has previously only been reported when hepatocytes were deliberately cocultured with other cell types.[17] What we have shown is that one formulation of biodegradable polymer material, films made from a 50/50 (w/w) blend of PLLA and PLGA 88:12, allows for good cell adhesion and for retention of a marker of differentiated function, albumin secretion, to the same degree as matrigel. The properties of the polymer which lead to this retention of function and why the blend is superior to the pure polymers are an area of future investigation. It is possible that the polymer surface acts to stabilize ECM proteins secreted by the cells in conformations which are more biologically relevant in comparison to polystyrene due to microphase separation of the crystalline PLLA from the amorphous PLGA. Such differences in protein conformation on surfaces have been reported for other substrates. For example, when the surface chemistry of glass is modified by derivatization with alkyl chains containing end groups of different polarities, there is little variation in the amount of fibronectin which adsorbs to the substrates but a dramatic variation in the behavior of cells on the adsorbed fibronectin, presumably due to differences in the conformation of the adsorbed fibronectin on the different substrates.[24] Surface roughness may also play a role. The blend films have a surface texture as shown in Figure 3, while the films of the pure polymers are smooth and untextured. Surface texture has been shown to modulate the morphology and differentiated state of other epithelial cell types in culture.[10]

It is significant that the polymer material does not require any intentional coating with exogenous biospecific molecules to allow maintenance of appropriate cell behavior. The most abundant source of proteins such as laminin, which enhance retention of differentiated function, is tumor tissue, and regulatory problems would likely be encountered for devices intended for human use. The coating procedure may also be technically difficult; it is not clear that the appropriate proteins would retain biologic activity if dried once they are adsorbed to the surface, and the device must be stored dry to prevent degradation. The results in the current study are the starting point for determining if other liver-specific markers of gene expression, such as cytochrome P450, are observed when hepatocytes are cultured on the films, and if the polymer material performs in a similar manner in vivo.

This work was supported by a grant from Neomorphics, Inc. The authors thank Mark Bulmer for excellent technical assistance.

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Biomaterials. Author manuscript; available in PMC 2011 January 1.

Published in final edited form as:
Biomaterials. 2011 January ; 32(1): 128–136. doi:10.1016/j.biomaterials.2010.09.006.

The Effect of Source Animal Age Upon Extracellular Matrix Scaffold Properties

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Abstract

Biologic scaffold materials composed of mammalian extracellular matrix (ECM) are commonly used for the repair and reconstruction of injured tissues. An important, but unexplored variable of biologic scaffolds is the age of the animal from which the ECM is prepared. The objective of the present study was to compare the structural, mechanical, and compositional properties of small intestinal submucosa (SIS)-ECM harvested from pigs that differed only in age. Degradation product bioactivity of these ECM materials was also examined. Results showed that there are distinct differences in each of these variables among the various age source ECM scaffolds. The strength and growth factors content of ECM from 3 week old animals is less than that of ECM harvested from 12, 26 or >52 week old animals. The elastic modulus of SIS-ECM for 3 week and >52 week old source was less than that of the 12 and 26 week source. Degradation products from all age source ECMs were chemotactic for perivascular stem cells, with the 12 week source the most potent, while the oldest source caused the greatest increase in proliferation. In summary, distinct differences exist in the mechanical, structural, and biologic properties of SIS-ECM harvested from different aged animals.

Keywords

Age/ageing; ECM (extracellular matrix); Mechanical properties; Bioactivity; Growth factors; Stem cell

INTRODUCTION

Biologic scaffold materials composed of mammalian extracellular matrix (ECM) are commonly used for the repair and reconstruction of injured or missing tissues and organs [1]. The mechanical and material properties of ECM scaffolds and the host tissue response to these biomaterials has been shown to depend upon the tissue specificity of the ECM material [2–4], processing methods [5–8], hydration [9], three dimensional configuration [10], and terminal sterilization [11,12]. The wide spectrum of clinical outcomes possible as a result of these factors

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has been recently reviewed for ECM scaffold materials commonly used for orthopedic applications [13].

Although the mechanisms of biologic scaffold remodeling and the associated outcomes are only partially understood, there is now convincing evidence that nascent growth factors [14, 15], surface topology and the distribution of surface ligands [16–19], modulation of the host innate immune response [5,20], and microenvironmental cues including mechanical loading [21,22] all contribute to the eventual functional outcome. It is generally believed that fetal wound healing results in less scar tissue formation and a more regenerative outcome than the adult response to tissue injury [23–25] and for this reason some biologic scaffolds use source materials that include fetal or neonatal cells [26,27], or fetal ECM [28]. The effect of donor animal age upon the structural and material properties of the ECM scaffolds produced from these tissues has not been systematically examined.

The objective of the present study is to compare selected structural, mechanical, and compositional properties of an ECM, specifically small intestinal submucosa ECM (SIS-ECM), harvested from pigs that differ only in age. We also investigate the response of select stem cells to the degradation products of these ECM materials.

MATERIALS AND METHODS

Overview of Experimental Design

SIS-ECM was prepared from the jejunum of pigs of 4 different ages: 3 weeks, 12 weeks, 26 weeks, and greater than 52 weeks. This SIS-ECM was subjected to mechanical and materials properties testing, analyzed for growth factor and glycosaminoglycan content, and evaluated for the mitogenic and chemotactic properties of the SIS-ECM degradation products.

Source and Preparation of ECM Material

The jejunum from Whiteshire Hamroc pigs of 4 distinctly different ages (3, 12, 26 and >52 weeks) were harvested immediately following euthanasia (Tissue Source, Lafayette, IN). The animals were of similar genetic heritage, and were raised and kept in identical husbandry conditions including diet and vaccination history. All tissues (intestines) were harvested on the same day and stored on ice prior to processing to create SIS-ECM as previously described [10,29]. Briefly, the intestines were rinsed with water and the mesenteric tissues were removed. The intestines were split longitudinally and mechanically delaminated to remove the tunica serosa, tunica muscularis externa, and the luminal portion of the tunica mucosa including most of the lamina propria. After delamination, the tunica submucosa and the basilar layer of the tunica mucosa including the muscularis mucosa and the stratum compactum of the lamina propria remained. The material was decellularized with 0.1% peracetic acid (Rochester Midland Corporation, Rochester, NY) followed by multiple rinses with saline and deionized water, and referred to as SIS-ECM. The SIS-ECM material was then frozen and/or lyophilized.

Preparation of SIS-ECM Degradation Products

SIS-ECM from the four age sources was powdered and digested at 10 mg/mL dry weight with 1 mg/mL pepsin (Sigma, St. Louis, MO) in 0.01N HCl for 48 h at 22°C before dialysis at 4°C in phosphate buffered saline using Slide-A-Lyzer Dialysis Cassettes, 3500 MWCO (Pierce, Rockford, IL). Protein concentration was quantified using the BCA protein assay (Thermo, Waltham, MA) against bovine serum albumin. The resultant material was referred to as SIS-ECM degradation products.

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Source of Cells and Culture Conditions

Perivascular stem cells were isolated by flow cytometry from human fetal muscle on the basis of expression of CD146 but not CD34, CD45 or CD56 [30]. Isolated cells were cultured in high glucose DMEM (Invitrogen, Carlsbad, CA.) growth medium containing 20% fetal bovine serum (Thermo), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma) at 37°C in 5% CO_2. These cells have been shown to have multipotent differentiation capacity and to be responsive to chemotactic and mitogenic properties of ECM degradation products [30,31].

Mechanical Testing

Lyophilized SIS-ECM from animals aged 12, 26 or >52 weeks was cut into longitudinal dog bone specimens using an identical template (midsubstance of 40 mm by 8 mm). SIS-ECM from 3 week old animals was narrower than the template and specimens were manually cut to the same length:width ratio of 5 with a mean width of 7.1 +/− 0.6 mm. Specimens were rehydrated for at least 48 h, after which time the width and thickness were measured at three locations prior to uniaxial tensile testing. Specimens were secured to tester grips by placing screws through holes in each end of the specimen to prevent slipping. All specimens were subjected to 10 pre-load cycles at 25.4 mm/min (0.100–0.500 N for SIS-ECM from animals aged 12, 26 or >52 weeks or 0.010–0.050 N for SIS-ECM from 3 week old animals) and immediately tested to failure, also at 25.4 mm/min. Peak load per unit width was calculated using mean width. Ultimate tensile stress was calculated from the peak load, mean width, and mean thickness: $\sigma=F/(w*t)$. Strain at failure was calculated by dividing the change in length by the original length: $\varepsilon=\Delta L/Lo$. Elastic modulus was taken as the tangent modulus (slope of the stress-strain curve) in the range of 20–60% of the ultimate tensile stress: $E=0.4*\sigma/(\varepsilon_{60\%\sigma}-\varepsilon_{20\%\sigma})$. Fourteen samples from each age source were tested.

Thickness Measurement

SIS-ECM from each age source was measured for thickness by a blinded operator applying equivalent compressive force (8.5 +/− 0.5 N) to all samples. Thickness after rehydration was measured by subjecting the specimens to lyophilization and then fully rehydrating the samples by soaking for at least 48 h before measurement by the same operator in the same fashion. Fourteen samples were measured for each age source.

Collagenase Digestion Assay

SIS-ECM from each age source was cut into 1×1 cm squares. Type 1 collagenase (Invitrogen) at 50 U/mL in phosphate buffered saline was added to 5 mg/mL dry weight SIS-ECM and incubated at 37 °C. Samples of the digestion solution were collected hourly and assayed for protein via the BCA protein assay against bovine serum albumin. Statistical comparison was performed at the 5 h time point. The experiment was repeated three times.

Growth Factor Analysis

SIS-ECM from each age source was powdered and suspended at 66 mg/mL in urea-heparin extraction buffer (2 M urea, 5 mg/mL heparin, 50 mM Tris, pH 7.4 and protease inhibitors 1mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM N-ethylmaleimide). The extraction mixture was rocked for 24 h at 4°C and then centrifuged at 12,000 g for 30 mins at 4°C. Supernatants were collected and 6 mL of freshly prepared urea-heparin extraction buffer was added to each pellet, re-incubated for 24 h at 4°C, re-centrifuged and supernatants collected. Supernatants were dialyzed against deionized water and protein concentration was quantified using the BCA protein assay against bovine serum albumin. Concentrations of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were determined with the Quantikine bFGF immunoassay and VEGF immunoassay (both R&D

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Systems, Minneapolis, MN) following the manufacturer's instructions. Each assay was performed in duplicate and the experiment repeated four times.

Protein Solubilization and SDS-PAGE

Powdered material from each age source was boiled for 10 mins at 2mg material per mL RIPA buffer containing 0.1% SDS, 1% sodium deoxycholate, and 1% NP-40 (Thermo). Solubilized protein was quantified using the BCA protein assay and 3 μg of protein from each age source was resolved on a 7.5% SDS-PAGE gel before staining with Silver Stain Plus (Bio-Rad, Hercules, CA).

Glycosaminoglycan Analysis

Sulfated glycosaminoglycan (sGAG) concentration in SIS-ECM from the different age source was determined using the Blyscan sGAG assay kit (Biocolor Ltd., Carrickfergus, United Kingdom). Samples were prepared by digestion of 50 mg/mL dry weight of each sample with 0.1 mg/mL proteinase K in buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM EDTA) for 48 h at 50°C. Digested samples were assayed following the manufacturer's instructions. The assay was performed three times. The spatial distribution of GAGs within the materials was examined by histologic methods. SIS-ECM from each age source was fixed in buffered 10% formalin, embedded in paraffin and cut into 5 μm sections, before deparaffinization and staining with Alcian blue at pH 2.5.

Cell Proliferation Assay

Perivascular stem cell proliferation in response to the presence of SIS-ECM degradation products was evaluated via 5-bromo-2′-deoxyuridine (BrdU) ELISA (Roche, Nutley, NJ). The cells were plated at 5×10^4 cells per well in a standard 96 well plate with 0, 2, 10 or 25 μg/mL SIS-ECM degradation products and BrdU and labeled for 18 h. Relative proliferation was quantified at 370 nm and 492 nm in a plate reader (Molecular Devices, Sunnyvale, CA). The assay was performed in triplicate on three occasions.

Metabolic Activity Assay

Perivascular stem cells were plated at 5×10^4 cells per well in a standard 96 well plate in medium containing 10% alamarBlue (Biotium, Hayward, CA) and 0, 2 or 25 μg/mL SIS-ECM degradation products. Metabolic activity was quantified fluorescently (Ex. 560 nm/ Em. 590 nm) over a period of 7 h and compared statistically at 7h. Each assay was performed in triplicate on three separate occasions.

Cell Migration Assay

The chemotactic response of perivascular stem cells to SIS-ECM degradation products was quantitatively evaluated using Neuro Probe 48-well micro chambers (Neuro Probe Inc., Gaithersburg, MD). Cells to be assayed were starved for 18 h in media containing 0.5% heat-inactivated FBS. The starved cells were harvested and resuspended in serum free media at a concentration of 6×10^5 cells/mL, and pre-incubated for 1 h. Polycarbonate PFB chemotaxis filters with a 8 μm pore size (Neuro Probe, Gaithersburg, MD) were coated with 50 μg/mL collagen I (BD Biosciences, San Jose, CA). The migration of 3×10^4 cells toward 0 or 100 μg/mL of SIS-ECM degradation products was tested for 3 h. Migrated cells were stained with Diff Quik (Dade AG Liederbach, Germany), and 3 magnification fields (20× objective) were counted from each well. Experimental conditions were tested in quadruplicate wells and the average number of migrated cells was determined for each condition. The experiment was repeated six times.

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Statistical Analysis

A one-way analysis of variance (ANOVA) was used to determine any differences between groups for each assay with a Tukey's post hoc test used to determine differences between pairs, with $p<0.05$ considered significant. For mechanical properties testing, data set outliers (no more than one value per group) were excluded when greater than two standard deviations away from the mean. All statistical analysis used SPSS Statistical Analysis Software (SPSS, IBM, Chicago, IL, USA).

RESULTS

Physical Characterization of SIS-ECM from Four Different Age Sources

Mechanical Analysis—Uniaxial tensile testing showed differences in SIS-ECM from the various age sources. SIS-ECM from 3 week old animals had lower peak load per unit width ($p<0.001$) compared to all other sources (Fig. 1a). SIS-ECM from 12, 26 and >52 week old animals failed at higher stress ($p<0.002$) compared to SIS-ECM from 3 week old animals (Fig. 1b). SIS-ECM from animals aged >52 weeks failed at higher strain ($p=0.016$) compared to SIS-ECM from 12 week old animals (Fig. 1c). SIS-ECM from 12 or 26 week old animals had higher elastic moduli ($p<0.018$) compared to SIS-ECM from 3-week-old animals, and SIS-ECM from 26 week old animals had a higher elastic modulus ($p=0.035$) compared to SIS-ECM from animals aged >52 weeks (Fig. 1d).

Thickness Measurement and Collagenase Digestion—The SIS-ECM material becomes substantially thicker with age. The 3 week old SIS-ECM was thinner ($p<0.001$) than all other ages, while the >52 week old SIS-ECM was thicker ($p<0.001$) (Fig. 2a). There was no statistical difference between the thickness of the 12 week old and the 26 week old SIS-ECM. Lyophilization and rehydration did not alter the thickness of any of the different age materials. The speed of remodeling *in vivo* and strength over time of an implanted device will in part be determined by the resistance of the device to enzymatic degradation. Samples of each age source material were incubated in collagenase and aliquots of solubilized protein collected hourly (Fig. 2b). SIS-ECM from all age source materials released protein from the material. The 3 and 12 week old source materials released more protein, more rapidly, than the older source materials ($p<0.005$). The >52 week old source material released less protein than any other source ($p<0.002$). After 24 h in the digestion solution the SIS-ECM from the 3, 12, and 26 week old source was no longer visible, while the >52 week material still appeared mostly intact.

Characterization of ECM Composition in Four Different Age Sources of SIS-ECM

Growth Factor Analysis and Glycosaminoglycan Quantification—Samples from each age source were analyzed for the concentration of growth factors bFGF and VEGF. SIS-ECM from the 3 week old source had less bFGF protein per mg dry weight of material ($p<0.001$) than any other age source (Fig. 3a). The 12 week source SIS-ECM had the greatest amount ($p<0.011$) with the amount of bFGF protein per mg dry weight material then decreasing with age (26 weeks less than 12 weeks p=0.011, >52 weeks less than 26 weeks $p=0.004$). The VEGF analysis showed that the 3 and >52 week source SIS-ECM had less VEGF protein ($p<0.001$) than the 12 or 26 week old source SIS-ECM, which had equivalent amounts of VEGF (Fig. 3b). No major differences in solubilized proteins were determined among the four different ages of SIS-ECM from SDS PAGE (Fig. 3c).

Samples from each age source were analyzed for the presence of sGAG (Fig. 3d). SIS-ECM from the 3 and 12 week old sources contained more sGAG per mg dry weight material ($p<0.001$) than the 26 or >52 week old source. The >52 week old source material SIS-ECM contained less sGAG than the 26 week old source material ($p=0.027$). The 3 and 12 week old

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source material contained equivalent amounts of sGAG. Staining of transverse tissue sections of SIS-ECM from each age source with Alcian blue to determine GAG spatial distribution showed that most of the GAGs are on the inner and outer edges of the material, with the 3 and 12 week old material (Fig. 3e and f respectively) showing more positive staining within material than the 26 or >52 week material (Fig. 3g and h respectively).

Mitogenic, Metabolic, and Chemotactic Properties of SIS-ECM on Perivascular Stem Cells

Degradation products derived from ECM are believed to play important roles in the recruitment and mitogenesis of selected cell types [32]. The effects of a pepsin digest of the different age source SIS-ECM materials on key processes of tissue remodeling, such as stem cell proliferation and recruitment, were therefore investigated. SIS-ECM degradation products at 2 μg/mL (black bars) from all age sources increased the proliferation of the stem cells ($p<0.001$) compared to untreated controls (Fig. 4). At 2 μg/mL, the SIS-ECM degradation products from the 26 and >52 week old source increased the proliferation of the perivascular stem cells more than the younger age source materials ($p<0.009$). SIS-ECM degradation products at 10 and 25 μg/mL also increased the proliferation of the perivascular stem cells ($p<0.010$) compared to the untreated control for all age sources except the 3 week old source material. The 26 and >52 week source materials increased the proliferation of the stem cells when compared to the 3 week old source material ($p<0.009$), but not the 12 week old animal source SIS-ECM. The 26 and >52 week old SIS-ECM degradation products caused an equivalent increase in proliferation at all concentrations tested.

The increase in metabolism of perivascular stem cells exposed to the different age sources of SIS-ECM degradation products was monitored for 7 h (Fig. 5). The SIS-ECM degradation products from all age sources increased the metabolism of the perivascular stem cells ($p<0.001$) at all concentrations tested. At 25 μg/mL (Fig. 5b), the SIS-ECM degradation products from the >52 week old source caused a greater increase in metabolism when compared to the SIS-ECM degradation products from 3, 12, or 26 week old animals ($p<0.001$). There was no difference in the increase in metabolism between the age sources of SIS-ECM degradation products at 2 μg/mL or between the 3, 12, or 26 week old animals at 25 μg/mL.

The SIS-ECM degradation products from all age sources are chemotactic for perivascular stem cells (Fig. 6, $p<0.001$). The migration of perivascular stem cells toward the SIS-ECM degradation products from 12 week old animals was increased over SIS-ECM degradation products from 3, 26, and >52 week old animals ($p<0.033$). The migration of cells toward SIS-ECM degradation products from >52 week old animals was less than for SIS-ECM degradation products from the other age animals ($p<0.001$). The migration of perivascular stem cells toward SIS-ECM degradation products from 3 and 26 old animals was equivalent.

DISCUSSION

Biomaterials composed of allogeneic or xenogeneic ECM are now commonly used for reconstruction of a variety of injured or missing tissues and organs [1]. However, clinical outcomes have varied considerably [13]. Many variables may contribute to these disparate outcomes, including the age of the animal from which the tissue is harvested. The results of the present study confirm that differences exist in the composition, structure, and mechanical properties of SIS-ECM prepared from tissues harvested from different aged animals. The present study represents the first systematic comparison of biologic scaffold materials that differ only in the age of the animal from which the materials were manufactured.

Ultimate stress, strain at failure, and elastic modulus of SIS-ECM in this study were similar to values obtained in other studies of SIS-ECM [33,34]. Single-layer SIS-ECM samples in this study and multi-layer SIS-ECM samples previously reported by other groups had a peak force

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per unit width within an order of magnitude of each other [35]. Peak force per unit width and elastic modulus of SIS-ECM in this study were also within an order of magnitude of some polymer surgical meshes [36], as was strain at failure [37]. Peak load per unit width and sample thickness were generally proportional to one another (Fig. 1a and Fig. 2a), the only exception being SIS-ECM from the oldest animals. The differences between the mechanical properties of SIS-ECM from animals aged >52 weeks and other age sources, was likely caused by changes in tissue organization because differences in protein composition between the sources appear to be subtle and were not consistent with the content of macromolecules such as growth factors and sGAGs (Fig. 3). The thickness of the SIS-ECM materials depends upon the technique of measurement. Under compressive force (8.5 +/−0.5N) we found that the materials have mean thicknesses between 12 (3 week source) and 33 (>52 week source) μm (Fig. 2a); however via histology we observed thicknesses ranging from 33 to 350 μm (Fig. 3e–h). The increased thickness observed via histology reflects the expansion of the material caused by histological processing. Previously reported values for SIS-ECM have been 100 [38] to 150μm [39].

There is a significant difference between the *in vitro* degradation rates of the different ages of SIS-ECM (Fig. 2b) which may have an effect upon the *in vivo* remodeling process of the device. The 12 and 26 week old source SIS-ECM degraded at different rates even though they had equivalent thicknesses (Fig. 2a). This indicates a change in the SIS-ECM, which is further continued in the >52 week source SIS-ECM. Age-related changes of ECM post synthesis were first reported in rat tail collagen fibers which underwent age related crosslinking [40] due to the Maillard reaction, a non-enzymatic modification of tissue proteins by reducing sugars. This process results in collagen fibers which are resistant to collagenase digestion [41,42]. A longer lasting implanted SIS-ECM device may be beneficial in some instances, and selection of the correct age or combination of SIS-ECM ages may allow the tuning of the device to a particular application.

The SIS-ECM prepared from all age sources modified the behavior of the perivascular stem cells, increasing their metabolism, proliferation and causing them to migrate towards the SIS-ECM degradation products (Fig. 4–6). These cells are believed to be a precursor for mesenchymal stem cells and therefore of particular interest because their multipotency and wide distribution throughout the body makes them a possible source for multiple different tissues that may require repair and regeneration [30,43]. The SIS-ECM biomaterials contain growth factors and sGAGs which are involved in cell signaling events and promote cell migration and proliferation [44–47]. The levels of growth factors and sGAGs reported here are very similar to levels previously reported for SIS-ECM [48]. After *in vitro* digestion with pepsin to generate the SIS-ECM degradation products it is unlikely that much if any active growth factor remains. The observed increase in proliferation, metabolism, and migration and of perivascular stem cells (Fig. 4–6) is likely caused by matricryptic peptides, derived from ECM during the process of degradation. These peptides can modulate biologic events including the recruitment of stem cells [49]. Such matrikines are generated as a result of ECM degradation and are a possible source of the effects seen here. Although the specific mediators of these events are not known, a head to head comparison of degradation products derived from the four different age sources of ECM shows that the >52 weeks age source has less potent chemotactic effects than the ECM from younger pigs, but both the >52 week and the 26 week age source result in greater mitogenic effects compared to the other age sources. This difference illustrates the complexities of cell-matrikine interactions and is likely to be dependent on proteinase-specific cleavage of ECM peptides during degradation.

The practical value of the findings herein depends upon the desired characteristics of the ECM device to be manufactured. For example, the design of scaffold materials that must withstand substantial mechanical loading after *in vivo* implantation and remodel into load-bearing or force-generating tissues are likely to require material properties that have the greatest ultimate

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tensile strength and elastic modulus. The present study shows that the animal from which the material is harvested should be at least 12 weeks of age but older animals provide no advantage with regard to this particular mechanical property (Fig. 1). Somewhat surprisingly, the pigs aged >52 weeks yield SIS-ECM with lower elastic modulus compared to 12 or 26 week old animal source. The *in vitro* degradation studies suggest that SIS-ECM scaffolds may persist longer *in vivo* if manufactured from animals aged >52 weeks (Fig. 2b). If bFGF and VEGF presence is a major concern, then the raw material should be harvested from animals at least 12 weeks of age (Fig. 3a and b), but the sGAG content is greatest in ECM prepared from the youngest age sources (3 and 12 weeks, Fig. 3d).

Although the methodology of the present study minimized the inherent biologic variability that exists in materials harvested from an animal source, there are limitations that must be noted in the interpretation of the results. First, the results represent the findings from a single source of ECM material; specifically porcine SIS-ECM. These results cannot necessarily be generalized to all ECM materials. Second, the mechanical and materials property testing was limited to uniaxial tensile testing. Other tests of mechanical and material properties, such as biaxial testing, ball burst strength, and suture retention strength may be more relevant for selected clinical applications and *in vivo* remodeling. Third, the *in vivo* biologic activity of ECM scaffold materials may not be related to *in vitro* tests of a particular stem cell type subjected to a mixture of ECM degradation products generated by pepsin digestion. However, in an effort to understand the variables that may affect a different clinical outcome, the isolation of a single variable, such as the age of the animal from which source material is harvested, is essential.

CONCLUSIONS

It is clear that the age of the source animal makes a difference to the properties of the SIS-ECM scaffold material. Selection of the appropriate age source material for a particular application should be a considered criterion. This study addresses one of the important variables to be considered in the design and manufacturing of biologic devices composed of mammalian ECM, the age of the source animal. The age of the animal from which the material is harvested has implications for the devices physical properties (Fig. 1 and 2), compositional makeup (Fig. 3), and the mitogenesis (Fig. 4), metabolism (Fig. 5), and migration (Fig. 6) of stem cells.

Future investigations in an animal model implanted with the different age sources of SIS-ECM are warranted, as well as experiments to determine the extent to which these findings can be generalized to other biologic scaffold materials.

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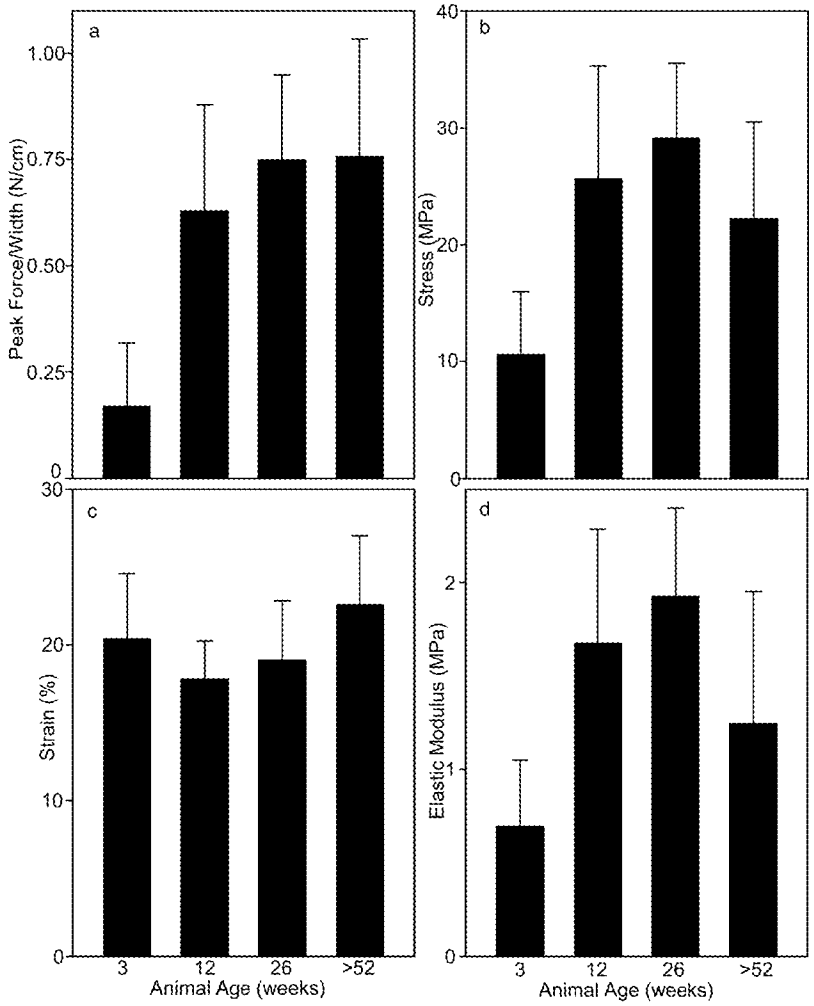


Fig. 1. Mechanical Properties of Porcine SIS-ECM Derived from Animals of Different Ages

Longitudinal SIS-ECM specimens were tensile tested to failure in uniaxial tension and mechanical properties were calculated from specimen dimensions and load-extension data. (a) Peak load per unit width was lower for SIS-ECM from 3 week old animals than all other sources. (b) Ultimate tensile stress was higher for SIS-ECM from 12 or 26 week old animals compared to 3 week old animals and higher for SIS-ECM from 26 week old animals compared to animals aged >52 weeks. (c) Strain at failure was higher for SIS-ECM from animals aged >52 weeks compared to 12 or 26 week old animals. (d) Elastic modulus was higher for SIS-ECM from 12 or 26 week old animals compared to 3-week-old animals and higher for SIS-ECM from 26 week old animals compared to animals aged >52 weeks. Data are means of 13 determinations with S.D.

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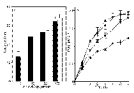


Fig. 2. Thickness and Collagenase Resistance of SIS-ECM from Different Age Sources

(a) SIS-ECM from the >52 week old source was thicker and 3 week old thinner than 12 or 26 week old source material. Thickness was measured at three locations for 13 samples from each age source. SIS-ECM from each age source which had never been lyophilized (black bars) or lyophilized and then fully rehydrated (white bars) was measured for thickness. Data are means with S.D. (b) SIS-ECM from older animals was more resistant to type I collagenase digestion. Squares of SIS-ECM material were digested with collagenase and the protein released into the digestion solution measured for 3 week old (circles), 12 week old (triangles), 26 week old (squares) or >52 week old (diamonds) source material. Data are means of triplicate determinations with S.D. The assay was performed on three occasions with similar trends observed.

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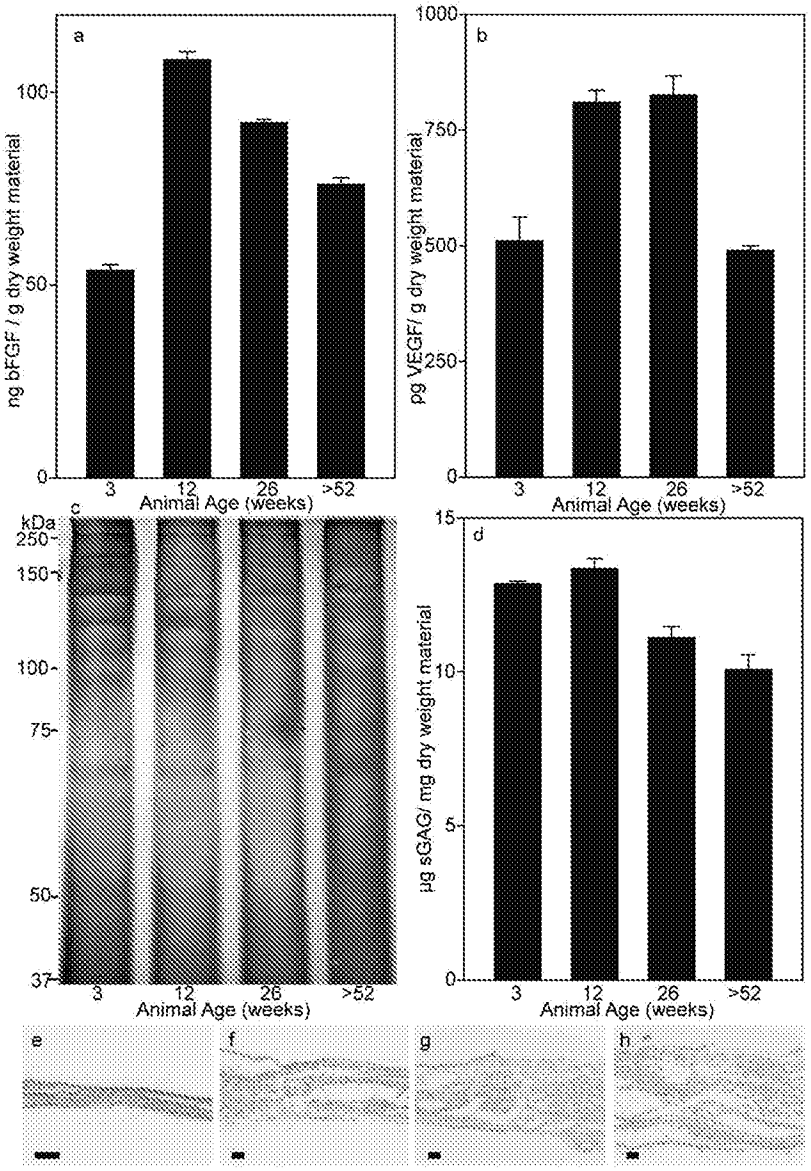


Fig. 3. Growth Factor and sGAG Quantification

(a) SIS-ECM from the 12 and 26 week old sources had more bFGF and (b) VEGF than the 3 or >52 week old source materials. Data are means of duplicate determinations with S.D. The assay was performed on four occasions with similar trends observed. (c) Protein from each different age source of SIS-ECM was solubilized and 3 µg was resolved via 7.5% SDS PAGE and visualized by silver staining. The pattern of protein bands shows no major differences between each age source. (d) SIS-ECM from 3 and 12 week old source animals had more sGAGs than the older animal source materials. Data are means of triplicate determinations with S.D. The assay was performed on three occasions with similar trends observed. (e–h) SIS-ECM was stained with Alcian blue to detect sGAGs from (e) 3 weeks, (f) 12 weeks, (g) 26 weeks and (h) >52 weeks source material. Black bar = 50 µm.

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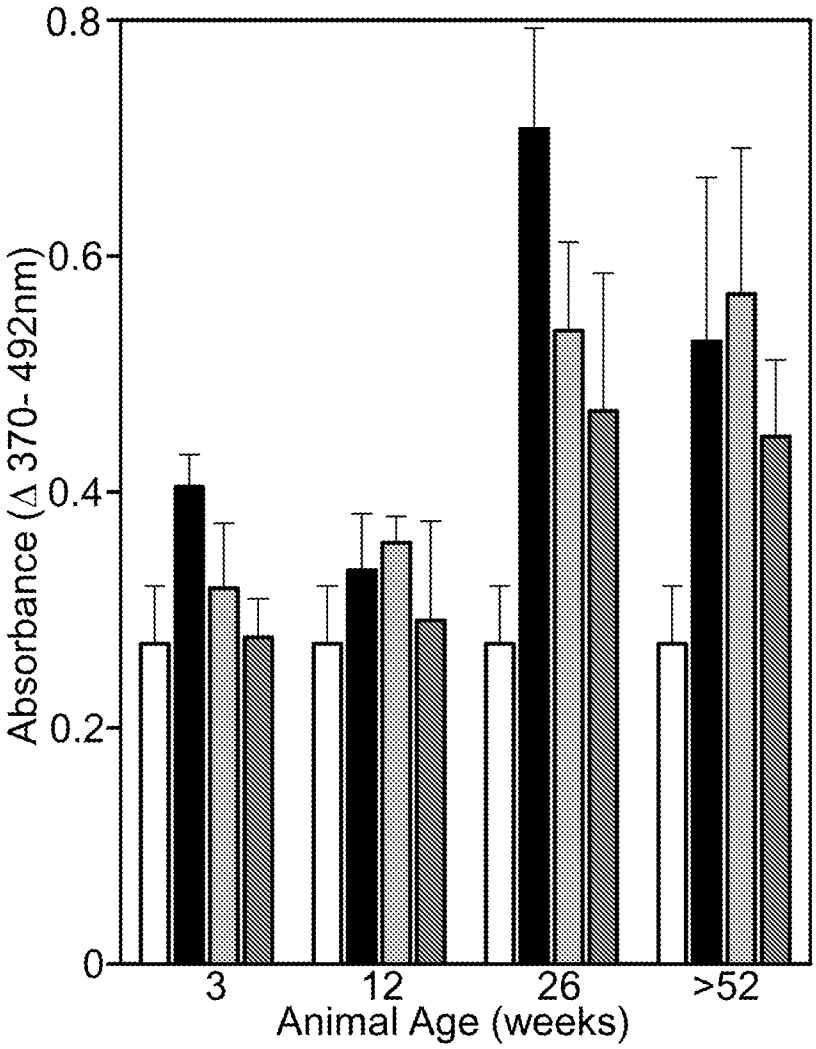


Fig. 4. Proliferation of Perivascular Stem Cells Exposed to Different Age Source SIS-ECM

SIS-ECM degradation products from all source materials increased proliferation, with the 26 and >52 week source material increasing proliferation the most. Perivascular stem cells were inoculated into media containing 0 (white bars), 2 (black bars), 10 (light grey bars), or 25 (dark grey bars) μg/mL SIS-ECM degradation products from the different age source materials and incubated for 18h. DNA synthesis was monitored via BrdU incorporation immunoassay and quantified colormetrically at 370 and 492 nm. Data are means of triplicate determinations with S.D. The assay was performed on three occasions with similar trends observed.

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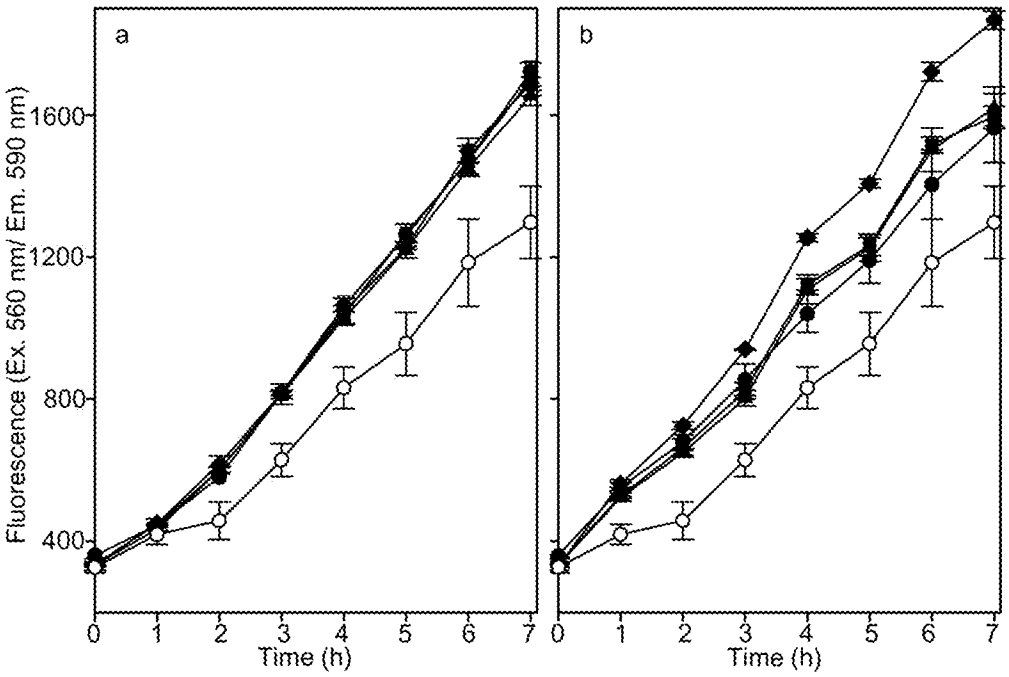


Fig. 5. Metabolism of Perivascular Stem Cells Exposed to Different Age Source SIS-ECM

SIS-ECM degradation products from all source materials increased metabolism, with the >52 week source material increasing metabolism the most. Perivascular stem cells were inoculated into media containing alamarBlue and (a) 2 μg/mL or (b) 25 μg/mL SIS-ECM degradation products from 3 week old (black circles), 12 week old (black triangles), 26 week old (black squares) or >52 week old (black diamonds). Perivascular stem cell metabolism without SIS-ECM (white circles) is shown as a control. AlamarBlue metabolism was quantified fluorescently (Ex. 560 nm/ Em. 590 nm) over 7h. Data are means of triplicate determinations with S.D. The assay was performed on three occasions with similar trends observed.

RegenX Science Exhibit 1062
Page 572

NIH-PA Author Manuscript

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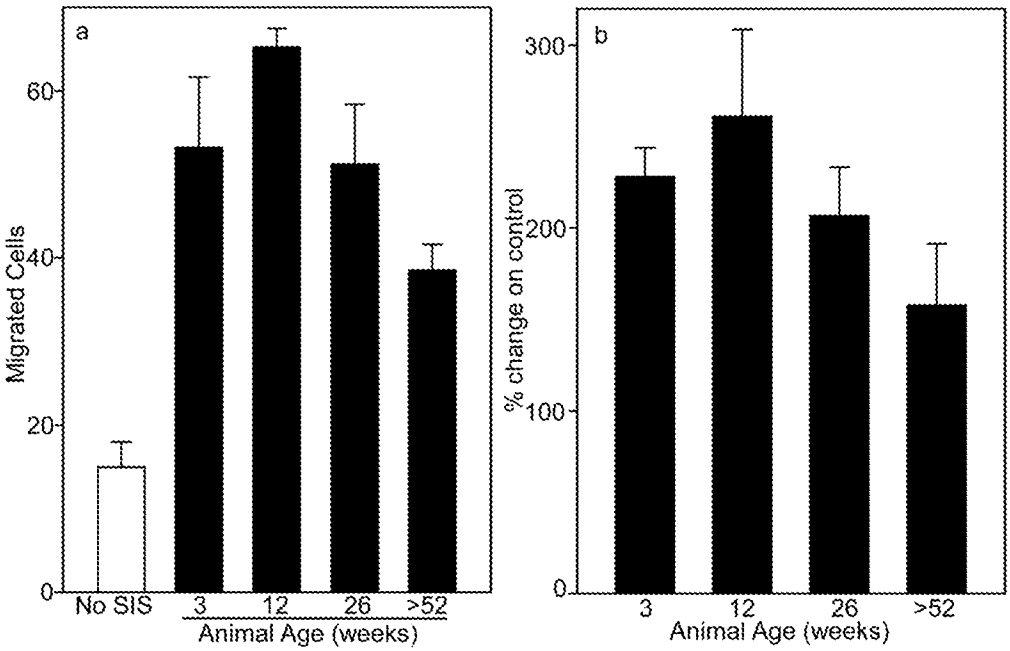


Fig. 6. Migration of Perivascular Stem Cells Exposed to Different Age Source SIS-ECM

The SIS-ECM degradation products from 12 week old animals caused greater migration and >52 week old animals less migration of perivascular stem cells than SIS-ECM from 3 or 26 week old animals. (a) The migration of perivascular stem cells toward 0 (white bar) or 100μg/mL (black bars) of the SIS-ECM degradation products from the different age source material was measured in a chemotaxis chamber for 3h. The y-axis represents the number of migrated cells in three 20× magnification fields. The data are means of quadruplicate determinations with S.D. The assay was performed on six occasions with similar trends observed. (b) Average of averages from six cell migration experiments showing fold migration change from 0 μg/mL SIS-ECM control. Error bars are S.D.

RegenX Science Exhibit 1062
Page 573

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	Ruth A. Davis
Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

Form PTO – 1449

Copies of cited references

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Kevin MacDonald/		
Printed name	Kevin MacDonald		
Date	November 11, 2016	Reg. No.	74,522

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: November 11, 2016 Signature: /Holly E. Johnson/ (Holly E. Johnson)

DOCKET NO.: S1860.70000US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	Ruth A. Davis
Art Unit:	1651

CERTIFICATE OF ELECTRONIC FILING UNDER 37 C.F.R. § 1.8

The undersigned hereby certifies that this paper, along with any paper referred to as being attached or enclosed, is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4), on the 11th day of November, 2016.

/Holly E. Johnson/
Holly E. Johnson

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed before the mailing of a first Office Action on the merits in the above-identified case.

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of

presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the enclosed search reports or other communications from a corresponding or related International or Foreign National Application:

Serial No.	Date of Mailing	Type(s) of Communication	Docket No.
EP 14737892.1	**September 28, 2016**	**Extended European Search Report**	**S1860.70000EP00**
PCT/US2014/010890	**May 15, 2014**	**International Search Report and Written Opinion**	**S1860.70000WO00**
PCT/US2014/010890	**July 23, 2015**	**International Preliminary Report on Patentability**	**S1860.70000WO00**

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Kevin MacDonald/_____
Kevin MacDonald, Ph.D., Reg. No. 74,522
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: S1860.70000US01
Date: November 11, 2016
xNDDx

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **11/09/2016**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 48	Minus	** 40	= 8	X $40 =	320
Independent (37 CFR 1.16(h))	* 11	Minus	***11	= 0	X $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	320

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
JAMES MASON

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Document code: WFEE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01	3358

23628 7590 01/25/2017
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
DAVIS, RUTH A	

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
01/25/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 14/759,895	Applicant(s) EARLY, RYANNE	
	Examiner RUTH DAVIS	Art Unit 1651	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *11/9/2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-48* is/are pending in the application.

5a) Of the above claim(s) *1-4,6-19,23-25 and 27-33* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *5,20-22,26 and 34-48* is/are rejected.

8)☒ Claim(s) *5 and 46* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Election/Restrictions

Applicant's election without traverse of Group II, claims in the reply filed on November 9, 2016 is acknowledged.

New claims 34 – 48 are drawn to the elected invention and are therefore being examined on the merits. Claims 1 – 48 are pending; claims 1 – 4, 6 – 19, 23 – 25 and 27 – 33 are withdrawn; claims 5, 20 – 22, 26 and 34 – 48 have been considered on the merits.

Claim Objections

Claim 5 and 46 are objected to because of the following informalities:

In claim 5, "urodele" should be capitalized.

In claim 46, there are two adjacent ",," in line 1.

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 20, 42 and 45 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

In claim 20, "ECM" should first be spelled, then followed by the abbreviation thereafter.

In claim 42, the phrase "a human agent" is not adequately defined by the claim language or specification.

Claim 45 is rendered indefinite because it is unclear if "particles of the Urodel ECM" is intended to be the form of the ECM, such as in particle or powdered form, or if the limitation is intended to mean that the implant contains different components of the ECM.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 5, 20 – 22, 34 – 41 and 44 – 47 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. Claims 5, 20 – 22, 34 – 41 and 44 – 47 are

directed to an extracellular matrix (ECM), or decellularized ECM, which are all products of nature. The claims do not include additional elements that are sufficient to amount to significantly more than the judicial exception because they read on components, such as collagen, that are not in any way significantly different than their natural counterparts in structure or function. The claims merely require such components removed from their natural habitat, without functionally or structurally changing them. Despite the hand of man required to effect such isolation, this alone is insufficient to materially change the individual components that would amount to significantly more than their natural counterparts.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.

Claim(s) 5, 20 – 22, 34 - 37, 45 and 46 are rejected under 35 U.S.C. 102a1 as being anticipated by Smith et al. (1982).

Smith teaches compositions comprising collagen (ECM components, decellularized ECM or fractions thereof) from Ambystoma tigrinum (a Urodele) (abstract, entire reference). Although Smith does not teach the process by which the collagen is produced, these limitations are considered to product by process type limitations. It is noted that the patentability of a product does not depend on its method of production. If the claimed product is the same or

obvious from a product in the prior art (i.e. the product disclosed in the cited reference), the claim is unpatentable even though the reference product was made by a different process. When the prior art discloses a product which reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C 102 and/or 35 U.S.C 103 are proper. (MPEP 2113) Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen.

Therefore, the reference anticipates the claimed subject matter.

Claims 5, 20 - 22, 34 - 39, 41, 44 – 47 are rejected under 35 U.S.C. 102a1 as being anticipated by Satoh et al. (2012).

Satoh teaches acellular collagen sheets (ECM components, decellularized ECM or fractions thereof) from axolotl (p.192). Although Satoh does not teach each of the claimed process steps by which the collagen is produced, these limitations are considered to product by process type limitations. It is noted that the patentability of a product does not depend on its method of production. If the claimed product is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference), the claim is unpatentable even though the reference product was made by a different process. When the prior art discloses a product which reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C 102 and/or 35 U.S.C 103 are proper. (MPEP 2113) Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen.

Therefore, the reference anticipates the claimed subject matter.

Claims 5, 22 and 47 are rejected under 35 U.S.C. 102a1 as being anticipated by Seifert et al. (2012).

Seifert teaches an ECM from axolotl. Regarding claim 22, the various skin samples disclosed therein are considered materials impregnated with, coated with or encapsulating, or having attached thereto, Urodele ECM.

Therefore, the reference anticipates the claimed subject matter.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 5, 20 – 22, 26 and 34 – 48 are rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

Asem teaches decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col.5 line 23-39), derived from amphibians (col.4 line 35-40). The ECM may in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col.6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col.4 line 30-35) or with cell culture comprising growth

factors for culturing human (col.11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col.7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45).

Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl. However, at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration.

Thus, the claims are rendered obvious.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on M-F 9:00 -4:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUTH DAVIS/
Primary Examiner, Art Unit 1651

Notice of References Cited	Application/Control No. 14/759,895	Applicant(s)/Patent Under Reexamination EARLY, RYANNE	
	Examiner RUTH DAVIS	Art Unit 1651	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-6,485,969 B1	11-2002	Asem; Elikplimi K.	C12N5/0068	435/1.1
	B	US-				
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Smith et al., "Collagens From the Skin and Cartilage of the Larval Salamander Ambystoma tigrinum", J of Exp Zoology, 220:243-250, 1982
	V	Satoh et al., "Collagen Reconstitution Is Inversely Correlated with Induction of Linb Regeneration in Ambystoma mexicanum", Zoological Science, 29: 191-197, 2012
	W	Seifert et al., "Skin Regeneration in Adult Axolotls: A Blueprint for Scar-Free Healing in Vertebrates", PLoS ONE, www.plosone.org, April 2012, Vol.7, Issue 4, e32875
	X	

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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	1	of	2	GROUP ART UNIT: 1651	EXAMINER: Ruth A. Davis

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Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
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OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
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THE JOURNAL OF EXPERIMENTAL ZOOLOGY 220:243-250 (1982)

Collagens From the Skin and Cartilage of the Larval Salamander *Ambystoma tigrinum*

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ABSTRACT The major collagens of larval salamander *(Ambystoma tigrinum)* skin and cartilage were purified and characterized by CM-cellulose chromatography and amino acid analysis of the component α chains. The collagens are similar to type I (skin) and type II (cartilage) collagens of birds and mammals. The type I molecule has the chain composition $(\alpha 1)_2\alpha 2$, and the type II molecule is an $(\alpha 1)_3$ molecule. Both collagens have lower levels of proline hydroxylation than the collagens isolated from homoiotherms, and generally higher serine contents. The type II collagen molecule has about half of its lysine residues hydroxylated, as in cartilage collagens of other species. Cyanogen bromide digestion of the component α chains followed by CM-cellulose chromatography or SDS gel electrophoresis of the peptides confirms that each chain is a different gene product and allows identification of the collagens in this species. Similar collagens were isolated from newt *(Notophthalmus viridescens)* tissues by pepsin extraction and fractional salt precipitation. These data will allow more effective utilization of the regenerating salamander limb for studies of collagen metabolism during regeneration.

INTRODUCTION

Limb regeneration in urodele amphibians offers a model system for observing and manipulating developmental events, including the breakdown and removal of collagenous structures in the stump (Grillo et al., '68; Dresden and Gross, '70) and the synthesis and deposition of collagen in blastemal tissues and in differentiating regenerates (Mailman and Dresden, '76; Linsenmayer and Smith, '75, '76).

Collagen is the major fibrous component of connective tissues, and is a potential marker for differentiation in salamander limbs. Collagen molecules are composed of three polypeptide chains, called α chains, tightly coiled into a triple helix. The α chains have a molecular weight of 95,000 and many common compositional features, such as a glycine residue in every third position, and both hydroxyproline and hydroxylysine residues. However, in birds and mammals, several genetically distinct collagens have been described that differ in α chain composition (Miller, '76). Skin, bone, and tendons contain type I collagen, with two identical subunits called α1(I) chains and a third, different chain called α2. It can be represented by the formula $[\alpha 1(I)]_2\alpha 2$. Type II collagen, $[\alpha 1(II)]_3$, is confined largely to cartilage. It is composed of three identical subunits called α1(II), chains which are different from both α1(I) chain and α2 chains in primary sequence. The third major interstitial collagen has three identical chains and is designated type III, or $[\alpha 1(III)]_3$. It is abundant in fetal skins and in internal organs. The α1(III) chain is again different in primary structure from the other collagen α chains, and furthermore, the three chains within a type III molecule are linked in disulfide bonds.

In birds and mammals, skin contains type I and type III collagens, and may contain other types (Kefalides, '69; Burgeson et al., '76; Chung et al., '76), whereas cartilage contains mainly type II collagen (Miller and Matukas, '69; Miller, '76). In the larval salamander, skin and cartilage collagens have been character-

This paper represents contribution number 886 from the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities.

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ized only by chain composition. We used radioactive collagens produced in vitro by explants of skin or cartilage to determine the α chain composition of the collagens produced by these tissues in larval *Ambystoma opacum, A. punctatum,* and *A. tigrinum* (Linsenmayer and Smith, '75, '76). Now, we have characterized chemical amounts of the major collagens of the salamander limb to allow more effective utilization of the regenerating limb system for the study of transitions in collagen types synthesized during differentiation of limb tissues, during blastema formation, and during experimentally induced scarring. We have confirmed that the major skin collagen in the larval salamander *(A. tigrinum)* is a type I-like molecule and that the major cartilage collagen is a type II-like molecule. The molecules can be identified by the behavior on gel electrophoresis and on CM-cellulose chromatography of the component α chains and the peptides derived from these preparations by cyanogen bromide cleavage.

MATERIALS AND METHODS

Preparation of collagens

Large (average wt = 8.2 g) larvae of *Ambystoma tigrinum* obtained from Michael Tolley, Donelson, Tennessee, were anaesthetized in a 1:1,000 solution of ethyl m-aminobenzoate methane sulfonate (Eastman), decapitated, and the skins and cartilages dissected free of other tissues and placed on ice. Subsequent manipulations were done at 4°C.

The skins were sequentially extracted in 0.4 ionic strength phosphate buffer, pH 7.6, and three changes of 0.5 M acetic acid. Each extract was clarified by centrifugation, and the collagen in the supernatant was purified as described previously (Gross, '58; Linsenmayer and Smith, '76). Collagens were precipitated from the neutral extracts by addition of 20% NaCl, then redissolved in 0.5 M acetic acid and precipitated by addition of 10% NaCl. Those extracted by acetic acid were first precipitated with 10% NaCl, then redissolved in 0.4 ionic strength phosphate buffer and precipitated with 20% NaCl. Finally, collagens from either extraction procedure were precipitated from potassium phosphate buffer by dialysis against 0.01 M Na_2HPO_4. These low ionic strength pellets were taken up in 0.1 M acetic acid, dialyzed, and lyophilized for further analysis. Collagen recovery was determined by hydroxyproline analysis (Woessner, '61).

The cartilages from the larval skeletons were minced, extracted with 2 M $MgCl_2$, and suspended in 0.5 M acetic acid. Pepsin (20 mg/g wet tissue) was added to the suspension, the pH was adjusted to 2.5 with HCl, and the digestion allowed to proceed at 4°C for 72 h. Then, the suspension was neutralized to inactivate the pepsin and dialyzed into potassium phosphate buffer, pH 7.6, ionic strength, 0.4. The neutral solution was clarified by centrifugation, and the collagen in the supernatant was purified as described above for the skin extracts.

The pepsin digestion technique was also applied to tissues of the newt *(Notophthalmus viridescens)* and to *A. tigrinum* skin residues following acetic acid extraction. Some samples were fractionated by precipitation from neutral buffer (Trelstad et al., '76) by dropwise addition of 4.4 M NaCl in water to collagen solution in 0.4 ionic strength phosphate buffer. At 2.2 M NaCl/0.2 ionic strength phosphate buffer, type I precipitates and type II remains in solution.

Collagens from the skin of tadpoles of the bull frog *(Rana catesbiana),* from chicken tissues, from rat tissues, and from rabbit tissues were prepared by similar methods and used for comparison throughout the study.

Analyses of the collagens

The purified collagens and the cyanogen bromide peptides derived from the collagens were analyzed by CM-cellulose chromatography on jacketed columns maintained at 42°C. Collagen solutions were denatured by heating for 10 min at 52°C and applied to 0.9 × 4 cm columns of CM-cellulose equilibrated with 0.02 M acetate buffer, pH 4.8. The component polypeptide chains were eluted at a flow rate of 50 ml/h with a superimposed linear gradient of NaCl from 0 to 0.14 M over a volume of 200 ml, as described previously (Piez et al., '63; Linsenmayer and Smith, '75). The column effluent was continuously monitored at 228 nm and fractions of 4 ml were collected. All buffer solutions contained 1 M urea. Recoveries were determined on several samples by hydroxyproline analysis and were 40–50%.

Cyanogen bromide peptides were prepared in sealed tubes by dissolving the collagens in 70% formic acid at 10 mg/ml, gassing the solutions with nitrogen for 5 min, and adding 12 mg/ml cyanogen bromide. After 4 h at 37°C, the peptides were desalted on Biogel P2 columns equilibrated either with 0.02 M formate/0.02 M NaCl, pH 3.8, for chromatography of the peptides or with 0.1 M acetic acid for ly-

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ophilization and gel electrophoresis.

The peptides were applied to a 0.9 × 12 cm column of CM-cellulose equilibrated with 0.02 M formate/0.02 M NaCl, pH 3.8, and eluted with a superimposed linear gradient of NaCl from 0 to 0.15 M over a total volume of 300 ml. The flow rate was 30 ml/h, and fractions of 5 ml were collected. Column effluents were monitored at 222 nm. For each collagen or α chain type, the chromatographic profile of the cyanogen bromide peptides was reproduced on 2–6 preparations without significant variation in pattern.

Some samples of collagen or collagen peptides were also separated by molecular sieve chromatography. Whole denatured collagens were chromatographed on Agarose A-5M in 1M $CaCl_2$/0.05 M Tris, pH 7.5. Peptides were chromatographed on Sephacryl S-200 in the same buffer.

Pooled fractions containing the chromatographic peaks from either type of chromatography were dialyzed against dilute acetic acid and lyophilized for further analysis.

Amino acid analysis was performed on a Beckman 121 amino acid analyzer, using a single column method (Kang, '72). Samples were hydrolyzed under nitrogen in constant boiling HCl at 108°C for 24 h.

Both whole collagen preparations and cyanogen bromide peptides were analyzed by electrophoresis on SDS-polyacrylamide slab gels. The buffer systems used were either Tris-glycine (Laemmli, '70) or Tris-borate (Neville and Glossman, '74). Gel concentrations were 7.5% for α chains and 15% for peptides.

Gels 0.075 cm × 14 cm × 10 cm were loaded and run overnight at 20 volts under constant voltage conditions. The gels were fixed in 30% methanol/10% acetic acid for four h, then stained with 2.5% Coomassie blue (Weber and Osborne, '69).

RESULTS

Type I collagen

More than 80% of the hydroxyproline containing proteins were consistently extracted from skin of *Ambystoma tigrinum* larvae with cold 0.4 ionic strength phosphate buffer, pH 7.6, followed by three changes of 0.5 M acetic acid. On SDS-polyacrylamide gel electrophoresis, the extracts contained materials that comigrated with standard collagen components from rabbits. Both neutral buffer and acid extracts contained apparent α1 chains, α2 chains, the covalently cross-linked dimers of either two α1 chains (β1,1) or one α1 and one α2 chain (β1,2), as well as trimers (γ) (Fig. 1). Each of the extracts contained variable ratios of β and α components. On CM-cellulose chromatography, the salamander skin collagen again exhibits peaks corresponding to α1; β1,1; β1,2; and α2 components (Fig. 2). Materials recovered from these peaks comigrate with the appropriate known collagen components on SDS gel electrophoresis. The complexity of these chromatograms and the relatively poor separation between β1,2 and α2 components preclude accurate assessment of α1/α2 ratios in these preparations. However, the triple helical portion of the native collagen molecule is resistant to limited pepsin digestion, and the covalent cross-links are located in short, nonhelical regions at the ends of the molecule. Thus, digesting native collagen molecules in cold, dilute acetic acid with pepsin (4°C, 18 h) removes the nonhelical cross-link regions and converts the β and γ components to α chains (Fig. 2). The α1/α2 ratio determined from CM-

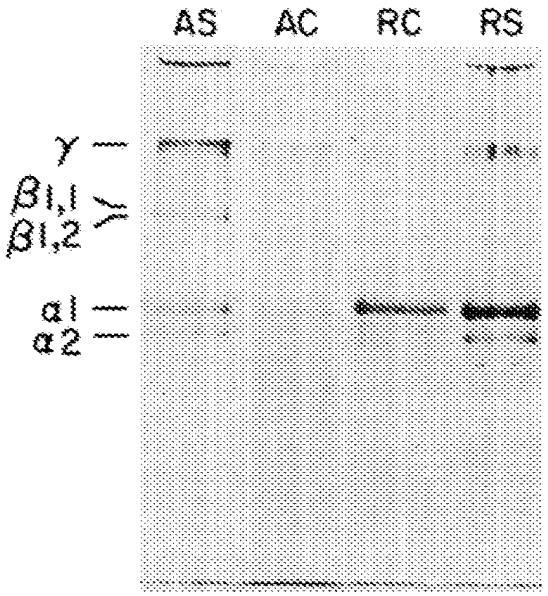


Fig. 1. SDS-polyacrylamide gel electropherogram of collagens from skin and cartilage of larval *Ambystoma tigrinum* plus reference collagens from rabbit tissues. Samples labeled AS and RS are from the skin of *Ambystoma tigrinum* and of the rabbit, respectively: AC and RC are from the cartilage of *A. tigrinum* and of the rabbit, respectively. The skin samples contain α1 and α2 chains, as well as the dimers composed of two α1 chains (β1,1) or one α1 and one α2 chain (β1,2). The cartilage samples contain largely α1 chains, although some samples have β1,1 and α components.

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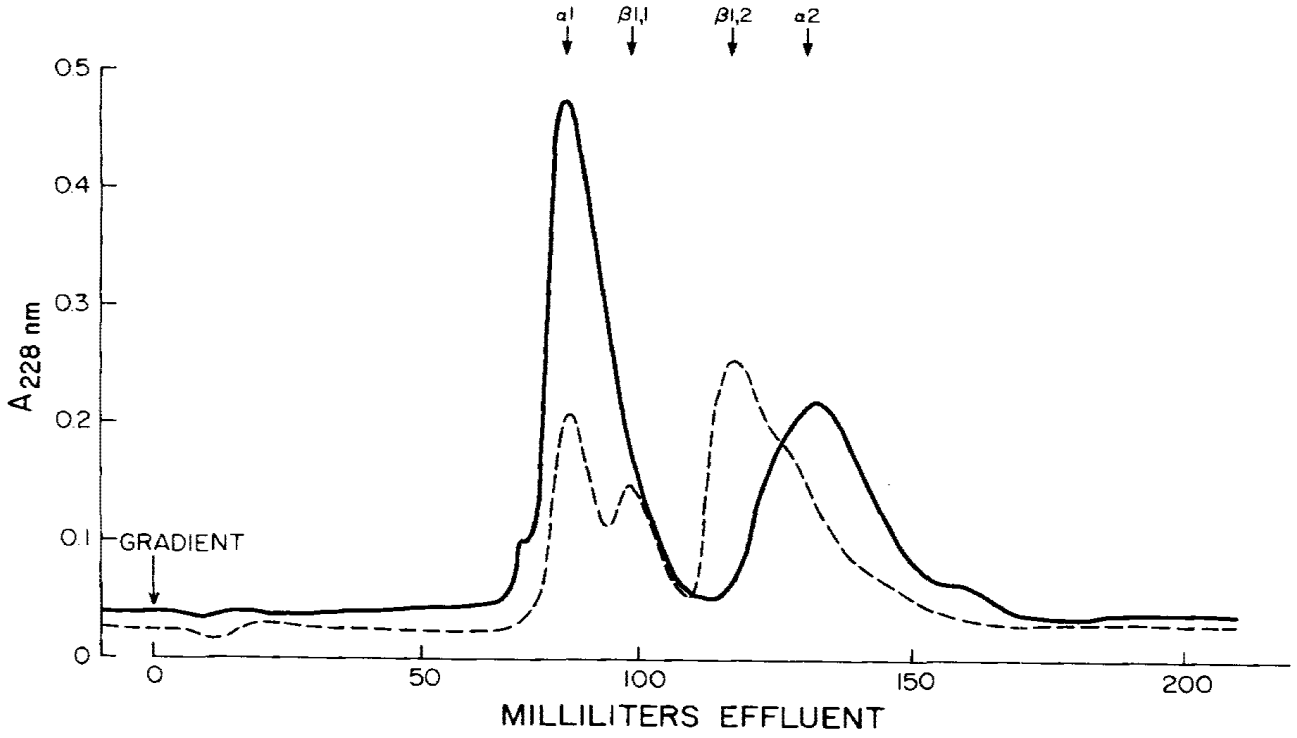


Fig. 2. Typical CM-cellulose chromatograms of collagen from skin of salamander larvae. The broken line is the chromatogram of the first extract with 0.5 M acetic acid. The peaks were identified as α1; β1,1; β1,2; and α2 by gel electrophoresis and amino acid analysis. The solid line is the profile of an aliquot of the acid extract after treatment with pepsin at 4°C for 18 h. Pepsin treatment under these conditions removes the nonhelical extension peptides from collagen, but does not attack the helical region of the molecule. Since the cross-links are located in the nonhelical ends, such treatment converts most of the β subunits to α chains. On this preparation, the resulting α1/α2 ratio was 1.9.

cellulose chromatograms on three such preparations was close to 2/1 (1.7–1.9) (Fig. 2).

The amino acid composition of α1 and α2 chains further purified and separated from each other by CM-cellulose chromatography, and from β components by gel filtration on Agarose A5 M, shows many similarities to the composition of type I α chains from birds and mammals (Table 1). The collagenous nature of the chains is confirmed by the presence of high levels of hydroxyproline and hydroxylysine, and their purity is confirmed by the absence of cystine and the presence of about one-third of the residues as glycine. The salamander α chains have lower levels of proline hydroxylation than are seen in the collagens of homoiotherms. When compared to the α1(I) chain of the chick, the α1 type I chain reveals a somewhat higher serine and lower alanine content. The α2 chain also shows a very high serine content. Both proline and lysine hydroxylation levels are lower in this chain than in the corresponding chain from chick skin.

Cyanogen bromide cleavage of the type I collagen yields peptides that can be partially resolved by CM-cellulose chromatography and on SDS gels. The α1 chain yields 5–7 peptides that can be resolved by chromatography (Fig. 3) and on SDS gel electrophoresis (Fig. 4). The α2 chain yields four poorly resolved peaks on CM-cellulose chromatography (Fig. 3).

Type II collagen

Cartilages from *Ambystoma* larvae, extracted with 2 M magnesium chloride to remove proteoglycan, then pepsinized, yield a collagen molecule similar to the type II collagen of birds and mammals. It contains only α1 chains as determined by CM-cellulose chromatography or by gel electrophoresis (Fig. 1). The amino acid composition (Table 1) reveals many similarities to the corresponding chains from other cartilagenous tissues. As previously noted for the skin collagen, the level of proline hydroxylation is lower than that seen in the

TABLE 1. Amino acid composition of collagen α chains from salamanders[a]

	Ambystoma tigrinum α1(I)	Chicken[b] α1(I)	Ambystoma tigrinum α2	Chicken[b] α2	Ambystoma tigrinum α1(II)	Notophthalmus[c] viridescens α1(II)	Chicken[b] α1(II)	Sturgeon[d] α1(II)
3-Hydroxyproline	—	—	—	—	1	2	1	2
4-Hydroxyproline	81	99	78.5	94	86	86	97	88
Aspartic acid	39	46	54	52	52	50	50	46
Threonine	11	18	24	20	23	24	23	20
Serine	53	27	67	29	32	49	28	38
Glutamic acid	83	83	78	64	88	91	93	89
Proline	127	118	114	116	117	118	113	118
Glycine	353	336	331	340	344	325	336	338
Alanine	109	127	103	100	82	78	102	106
Cystine/2	0	0	0	0	0	0	0	0
Valine	11	16	20	31	16	22	23	14
Methionine	8	6	4	3	7	7	6	4
Isoleucine	6	7	8	18	9	10	9	8
Leucine	16	20	25	30	29	30	26	27
Tyrosine	—	2	3	3	2	4	1	1
Phenylalanine	13	13	13	14	14	10	12	12
Hydroxylysine	7	7	6	13	21	20	18	19
Lysine	29	25	24	17	21	12	17	16
Histidine	3	2	7	7	4	5	2	3
Arginine	50	47	44	49	53	51	43	51

[a]Values are given as residues/1,000 and are the means of three determinations. The α chains were purified by chromatography on CM-cellulose and agarose A-5M.
[b]Chick α chain compositions are from Trelstad et al. ('72).
[c]Newt α1 (II) collagen was isolated by fractional salt precipitation from pepsin extract of whole newt carcases.
[d]Sturgeon type II α chain composition is from Miller and Matthews ('74).

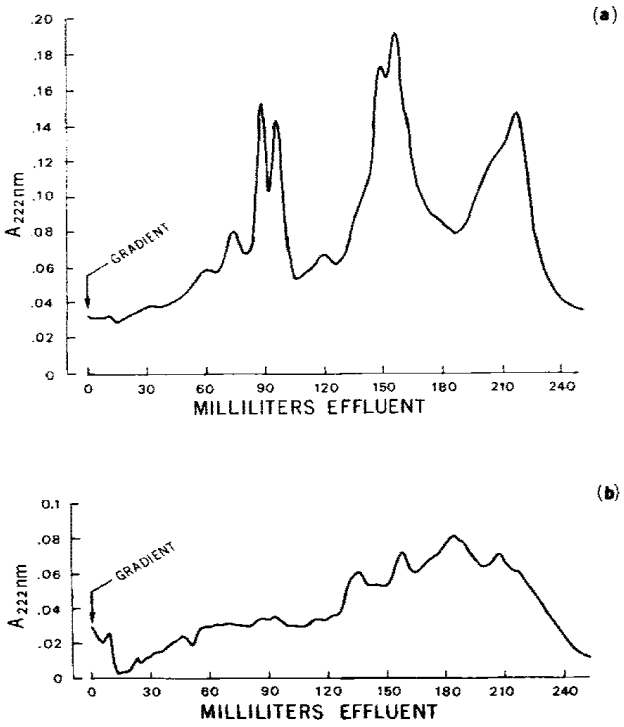


Fig. 3. Typical CM-cellulose chromatograms of cyanogen bromide peptides from (a) α1 and (b) α2 chains of salamander skin. The profiles are reproducible and characteristic for each chain. Because all of the α2 peptides elute before the last α1 peptide, it is advantageous to separate the α chains before cyanogen bromide digestion.

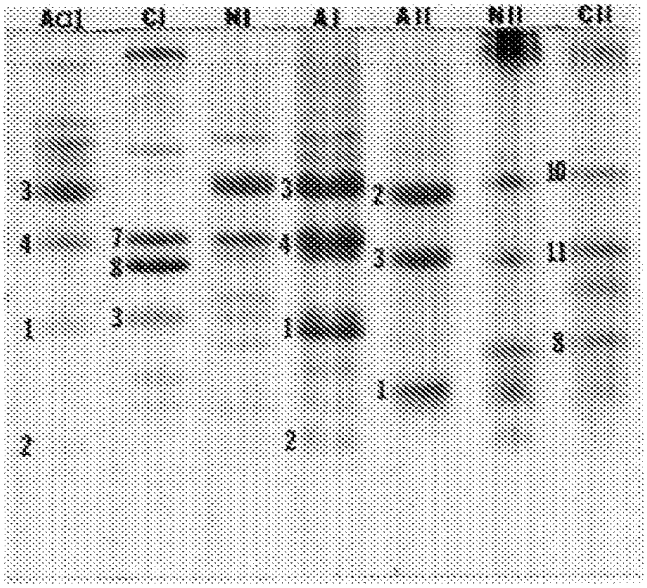


Fig. 4. SDS-polyacrylamide gel electropherogram of cyanogen bromide peptides of several amphibian collagens plus reference collagens from chickens. Samples labeled CII and CI are from chick α1(II) and α1(I) chains, respectively; AI and AII are from the homologous salamander collagens; NI and NII are from the homologous collagens of the newt; and Aα1 is from an α1 chain from salamander skin collagen. The numbers to the left of CI and CII are the published designations of the chicken α1 (I) and α1(II) peptides (Miller, '76). The numbers to the left of the salamander collagens correspond to the numbered peaks in Figure 5.

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collagens of homoiotherms. However, the hydroxylysine content of the salamander type II is as high as or higher than the levels reported in birds and mammals. These high levels of lysine hydroxylation and very low alanine content distinguish this collagen from skin collagens.

Cyanogen bromide digestion of α(II) followed by CM-cellulose chromatography yields a peptide map different from that produced by type I (Fig. 5), indicating that the collagen of the cartilage is a different gene product. The peptides resolved by gel electrophoresis (Fig. 4) are also unique. Direct digestion of the cartilage by cyanogen bromide yields a similar peptide map on either gels or CM-cellulose chromatography, confirming that type II collagen is the predominant collagen type in this tissue.

The newt, *Notophthalmus viridescens,* also has type II collagen (Fig. 4, Table 1) with properties similar to this collagen in larval salamanders, birds, and mammals. This molecular species was obtained through fractional salt precipitation of pepsin digests of newt tails and limbs. Type I collagen is insoluble in 2.2 M NaCl/0.2 ionic strength phosphate, pH 7.6, whereas type II remains in solution.

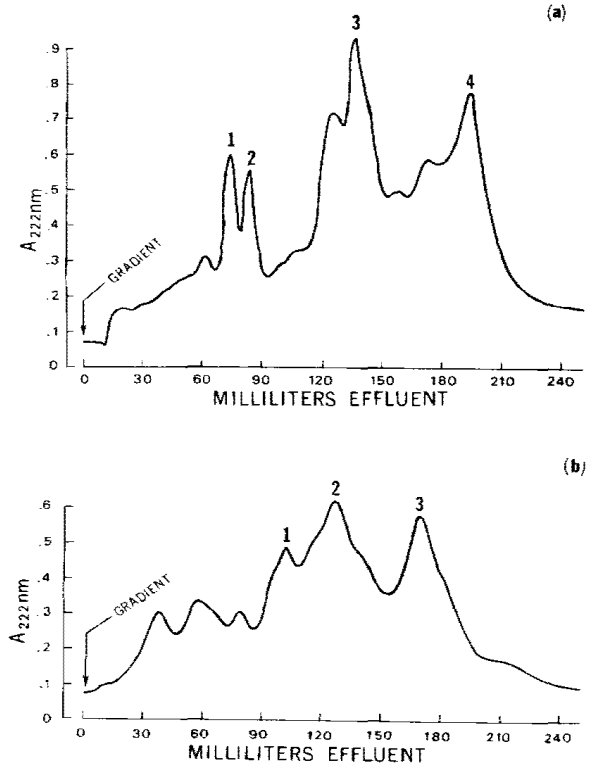


Fig. 5. Typical chromatographic profiles of (a) skin and (b) cartilage collagen from *A. tigrinum* larvae. The profiles are unique and reproducible. The major peptides from the numbered peaks are marked on Figure 4.

DISCUSSION

Because type II collagen is the major collagen type produced by differentiated hyaline chondrocytes, it has been extremely useful both as a marker for differentiation during development (Linsenmayer et al., '73a, '73b; von der Mark et al., '76) and in the analysis of chondrocyte phenotypic expression in vitro (Mayne et al., '75, '76, '77; Benya et al, '78; Benya and Nimni, '79). Furthermore, the surprising observation that embryonic chick neural retina synthesizes type II collagen for export to the vitreous body (Smith et al., '76; Newsome et al., '76) and that the corneal epithelium also deposits both type I and II collagen in the primary corneal stroma (Linsenmayer et al., '77; von der Mark et al., '77) suggests wider distribution and additional, as yet unknown, functions for this collagen in development. Thus, characterization of the collagen of salamander skin and cartilage will facilitate similar studies in the amphibian developmental systems, particularly in regeneration.

We had previously reported that the collagen produced by cartilages incubated in vitro with ^{3}H-proline had the α chain composition $(\alpha_1)_3$, whereas the molecule produced by skin had the chain composition $(\alpha_1)_2\alpha_2$ (Linsenmayer and Smith, '75; '76). The existence of these chains has been confirmed with chemical amounts of collagen extracted from the tissues. Furthermore, the suggestion that each type of α chain is a product of different genes has been verified by amino acid analysis and cyanogen bromide peptide mapping. By analogy to the collagens of birds and mammals, we can now designate the skin molecule type I, or $[\alpha1(I)]_2\alpha2$, and the cartilage molecule type II, or $[\alpha1(II)]_3$.

The type I molecule has a number of similarities to type I collagen from homoiotherms, including α1 and α2 chains as defined by their behavior on CM-cellulose chromatography and SDS gel electrophoresis, β1,1 and β1,2 components apparently cross-linked in nonhelical regions of the molecule, and the α1/α2 ratio of 2/1. The strongest evidence that the ratio of α1/α2 in type I collagen from *Ambystoma* larvae is 2/1 comes from our previous studies with radioactive collagens. In these experiments, β-aminopropionitrile was included in the medium to prevent cross-link formation. Thus, no β components formed and the CM-cellulose chromatograms were easily evaluated. Both skin preparations and the undifferentiated

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blastemal tissues reproducibly synthesized collagens with an $\alpha 1/\alpha 2$ ratio of 2/1. The ratios we obtained following pepsin digestion confirm the data from the studies in vitro.

The demonstration that the collagen found in the cartilage of larval urodeles is a product of different genes than the collagens of skin is of importance to both developmental studies and to phylogenetic considerations. During limb regeneration, chondrocytes are freed from the cartilage matrix and enter the blastema (Hay, '66). They lose their histological identity, proliferate as mesenchyme-like cells, then redifferentiate (Steen, '68). These changes that occur in vivo are similar in appearance to changes seen in chick and mammalian chondrocytes cultured in vitro. However, when we determined the chain composition of collagens produced by blastemal tissues derived in part from cartilage, we could detect only $(\alpha 1)_2\alpha 2$ molecules (Linsenmayer and Smith, '75, '76). In contrast, both chick and rabbit chondrocytes that lose chondrocyte morphology in vitro alter their collagen production from type II to include both type I, $[\alpha 1(I)]_2\alpha 2$, and a previously undetected collagen, the type I trimer, $[\alpha 1(I)]_3$ (Mayne et al., '75, '76, '77; Benya et al., '78; Benya and Nimni, '79). Investigation of the products of chondrocyte cultures derived from *Ambystoma* larvae should now allow verification of type I, type II, and type I trimer production. If chondrocytes that lose their histological identity in vitro are biochemically similar to those chondrocytes that lose their histological identity in vivo to enter the regeneration blastema, then these cultures should produce only $[\alpha 1(I)]_2\alpha 2$ collagen. If these cells produce $[\alpha 1(I)]_3$, then the loss of chondrocyte characteristics in vitro is a different process from the same loss in vivo.

At the phylogenetic level, salamander collagens have several features that distinguish them from the collagens of birds and mammals. Proline hydroxylation is uniformly lower than the levels seen in homoiotherms. Since the melting points that have been determined for newt collagen (Mailman et al., '74) are lower than the melting points found with mammalian collagens, the low level of proline hydroxylation is consistent with the proposition that the thermal stability of the collagen triple helix is proportional to hydroxyproline content (Ramachandran and Ramakrishnan, '76). As has been the case with other collagens from poikilotherms, lower hydroxylation of proline is accompanied by higher serine content (Rudall, '68; Pikkarinen and Kulonen, '69). Similar compositional features were also noted in the native collagen molecules extracted from newt *(Notophthalmus viridescens)* skin by dilute acetic acid (Mailman et al., '74) and from newt tails by limited pepsin digestion (Baum et al., '74).

The remaining differences between the amino acid content of collagens of the salamander and those of birds and mammals are slight. It is notable that lysine hydroxylation in the type II molecule is not lowered. Both the amphibian cartilage collagens studied here and the sturgeon cartilage and notochord type II molecules previously reported (Miller and Matthews, '73) have at least half of their lysine residues hydroxylated. This fact, and the conservation of the tissue distribution of type I and type II collagens in all vertebrates yet characterized, suggest functional advantages for these collagen molecules that are not yet fully understood.

Finally, although we have characterized the major collagens from skin and cartilage, other collagen types certainly exist. We have partially characterized two additional chains from salamander skin that have the electrophoretic mobility of the A and B chains thought to be associated with basement membranes in humans (Burgeson et al., '76; Chung et al., '76). The salamander chains also have high levels of hydroxylysine and measurable amounts of 3-hydroxyproline, features that are consistent with a basement membrane origin. We did not find any type III-like molecules in our extracts of skin, nor did we see any evidence of type III production in the radioactive collagens studied previously (Linsenmayer and Smith, '76). However, no systematic search of the 5% of the skin collagen that remains insoluble following pepsin extraction nor of the hydroxyproline containing molecules lost during purification has yet been made. This type of search has yielded evidence of at least three new chains in human cartilage (Burgeson and Hollister, '79) and was necessary to clearly identify type III collagen in chickens (Hermann and von der Mark, '77).

ACKNOWLEDGMENTS

We appreciate the support and helpful discussion of Dr. Jerome Gross and Dr. Andrew H. Kang, as well as the helpful discussion and interest of Dr. Robert L. Trelstad, Dr. Bryan P. Toole, and Dr. Jerome M. Seyer.

We would also like to thank Mr. Kenneth Shaw, Ms. Eileen Gibney, and Ms. Genevieve Fridland for excellent technical assistance.

Supported by National Institutes of Health grant AM 3564 and by funds from the Veterans Administration.

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
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ZOOLOGICAL SCIENCE **29**: 191–197 (2012)
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Collagen Reconstitution Is Inversely Correlated with Induction of Limb Regeneration in *Ambystoma mexicanum*

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Amphibians can regenerate missing body parts, including limbs. The regulation of collagen has been considered to be important in limb regeneration. Collagen deposition is suppressed during limb regeneration, so we investigated collagen deposition and apical epithelial cap (AEC) formation during axolotl limb regeneration. The accessory limb model (ALM) has been developed as an alternative model for studying limb regeneration. Using this model, we investigated the relationship between nerves, epidermis, and collagen deposition. We found that *Sp-9*, an AEC marker gene, was upregulated by direct interaction between nerves and epidermis. However, collagen deposition hindered this interaction, and resulted in the failure of limb regeneration. During wound healing, an increase in deposition of collagen caused a decrease in the blastema induction rate in ALM. Wound healing and limb regeneration are alternate processes.

Key words: limb regeneration, epidermis, nerve, accessory limb model, *Sp-9*, collagen

INTRODUCTION

Urodele amphibians have powerful regenerative capabilities, as they can regenerate missing body parts, including limbs. Limb regeneration begins immediately after amputation. The surrounding epidermal cells migrate and cover the amputation surface (Carlson et al., 1998). An interaction between the nerves and epidermal cells covering the amputation surface then occurs, resulting in the induction of a functional epithelial tissue called the apical epithelial cap (AEC). In the presence of nerves and AEC, undifferentiated cells called blastema cells emerge from differentiated tissues (Satoh et al., 2007; Satoh et al., 2010a). These blastema cells accumulate around the amputation plane and form a regeneration blastema that undergoes limb developmental processes to restore the original structure (Bryant et al., 2002; Gardiner et al., 2002). Although the mechanisms of limb regeneration are beginning to be understood, the induction process of blastema cells has not been well elucidated. Undifferentiated blastema cells are induced from differentiated tissues by a process called dedifferentiation. Dedifferentiation is a key event during the early phases of limb regeneration, highlighting the need to further investigate the early phases of limb regeneration.

Nerves are essential for limb regeneration, and neurogenic limbs become nonregenerative when nerves are removed (Wallace, 1981). In addition, denervation during an early phase of limb regeneration terminates limb regeneration, even when in progress (Singer, 1952). Thus, nerves play an important role in limb regeneration. The effects of nerves on limb regeneration have been investigated, and some factors, such as the nerve trophic factor, have been reported (Brockes and Kintner, 1986; Mullen et al., 1996; Wang et al., 2000; Dungan et al., 2002; Nye et al., 2003; Satoh et al., 2008b). Furthermore, a focus has been on these factors and the presumptive effects of nerves on the blastema mesenchyme. However, the neural regulation of the blastema epithelium has not been well studied. Recently, a new study model—the accessory limb model (ALM)—was established in a limb regeneration study (Endo et al., 2004). This model reveals an interaction between the nerves and wound epithelium during the early phases of limb regeneration (Satoh et al., 2008b; Satoh et al., 2009). Fibroblast growth factors (FGFs) are secreted by nerves and received by the overlying epithelium (Satoh et al., 2008b). FGF-exposed epithelium is specified as AEC, an essential component in limb regeneration, because its removal results in the failure of limb regeneration (Thornton, 1957; Wallace, 1981). Undifferentiated blastema cells are induced in the presence of AEC and nerves (Satoh et al., 2008a; Satoh et al., 2010a). The induced blastema cells are also maintained by the nerves and AEC. If nerves are eliminated from a regenerating limb, the blastema stops growing and starts differentiating or regressing, depending on the phase of limb regeneration (Singer, 1952).

As described above, ALM is an alternative model for limb regeneration studies and is suitable for studying the involvement of nerves in limb regeneration. Only nerve deviation and skin wounding are enough to induce a completely patterned limb without limb amputation (Endo et al., 2004). Skin wounding without nerve deviation results in simple skin regeneration in axolotl. The induced limb is called an acces-

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doi:10.2108/zsj.29.191

sory limb. An axolotl cannot move the accessory limb, although it has the same histology as a regular limb. Furthermore, during the process of limb regeneration in ALM, the same gene expression pattern can be seen as during regular limb regeneration and development (Satoh et al., 2007; Satoh et al., 2008b; Satoh et al., 2009). Moreover, the induced ALM blastema shows the same features as a regular blastema grown on an amputated limb (Satoh et al., 2007; Satoh et al., 2010a). ALM has several advantages compared to the regular limb regeneration model using the amputated limb, such as easier comparison of wound healing and limb regeneration. In an amputated limb, limb regeneration responses begin after amputation. When wound healing is needed for comparison, repeated denervation treatment appears to be necessary to inhibit limb regeneration and induce wound healing (Petrosky et al., 1980). Furthermore, the tissue types at the amputation surface are diverse, and each tissue drives its own healing process. Thus, wound healing in the regular model is not simple. In contrast, in ALM, only skin wounding and nerve deviation are sufficient to induce the blastema. Wounding without nerve deviation results in skin healing, without any damage to the deeper tissues such as muscles (Endo et al., 2004). Thus, using this model, comparison of wound healing and regeneration becomes easier. Furthermore, a completely patterned limb in ALM is reconstituted by a single regeneration system (AEC-dependent regeneration), while that in a regular limb regeneration model requires two different types of regeneration systems (AEC-dependent and -independent regeneration) (Satoh et al., 2010b). The major feature of ALM is limited tissue contribution. Thus, ALM reduces the complexity of limb regeneration.

In this study, we used ALM and focused on the involvement of nerves in AEC induction as well as the inhibitory effect of collagen deposition, which is synthesized during wound healing. Our results suggest that the regulation of collagen deposition by AEC and the nerves is important during limb regeneration.

MATERIALS AND METHODS

Animals and surgery

Animals that were 10–15 cm (nose to tail) in length were obtained from personal breeders and raised at 22°C. For ALM surgery, animals were anesthetized with MS-222. After surgery, they were put on ice in order to attach the rerouted nerve to the limb tissue. Regular limb regeneration surgery was performed according to a previously described protocol with some modifications (Endo et al., 2004). In brief, to reroute the nerve under intact skin, we first pulled the nerve out from a small incision made in the dorsal region of the upper arm. Then, a second small incision was made in the anteroventral region. A small tunnel was made with a fine forceps, and the nerve was pulled out through the tunnel from the dorsal to anteroventral region. The excess length of the nerve over the incision was cut, providing tension to the nerve. Because of this tension, the nerve was slightly smaller than normal. As a result, the nerve ending was located in the anterior region. The anterior epidermis was scraped with a syringe needle (27G; Terumo) to reroute the nerve to the skin collagen layer, and the nerve was pulled through the small incision. The nerve was subsequently placed on the exposed collagen. For nerve rerouting on specific days after skin wounding, a skin wound was first created on the anterior side of the upper limb, and the nerve was rerouted as described above. The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, and then incubated in water with 0.01% Tween 20, PMSF (1.74 μg/ml), and glutaraldehyde (20.18 μg/ml) for two days, resulting in an acellular ECM sheet. The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 37°C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed on the rerouted nerve.

Histology and immunohistochemistry

Samples were fixed overnight in 4% paraformaldehyde diluted with PBS. Next, the samples were incubated overnight in 10% EDTA for decalcification, and then in 30% sucrose at 4°C. The samples were then embedded in OCT compound (Sakura). A cryostat (1850; Leica) was used to cut 10-μm thick sections. Histological procedures were identical to those described previously (Satoh et al., 2008b).

We used anti-acetylated tubulin (Santa Cruz) and type I collagen antibody (Sigma) to visualize axons and collagen, respectively. Antigen retrieval was performed for collagen staining by incubating the sections in 5 μg/ml proteinase K in Tris-buffered saline with 0.01% Tween 20 for 30 min at room temperature, as described previously (Satoh et al., 2008a).

RT-PCR and in situ hybridization

Total RNA was prepared using the TriPure reagent (Roche). Reverse transcription was performed with Superscript III (Invitrogen). The following primers were used for RT-PCR: Sp-9 forward, 5′-GTGTGCAACTGGCTCTTCTG-3′; Sp-9 reverse, 5′-TGGGTTTT-GATGTGTTTGCT-3′ and Ribosomal protein L19 forward, CATG-GGCACTGGTAAGAGAAAAG; Ribosomal protein L19 reverse, GCGGCGCAAGATTCTCAT. In situ hybridization followed previously published procedures (Satoh et al., 2007).

RESULTS

Direct interaction between the nerves and epidermis resulted in *Sp-9* activation

A previous study of ALM revealed that an interaction between the nerves and wound epithelium is important for *Sp-9* activation (Satoh et al., 2008b). However, it was unclear whether a physical interaction was necessary to induce *Sp-9* expression. To investigate this, we deviated nerves under or on the dermal collagen layer (Fig. 1). When nerves were deviated under the dermal collagen layer (Fig. 1A), the direct interaction between the nerves and epidermis was inhibited. Axon ends could be seen under the dermal collagen layer (Fig. 1A, asterisk), but AEC formation was not observed histologically in the overlying epidermis (Fig. 1B), suggesting that direct interaction between the nerves and epidermis is necessary to induce AEC. To investigate this further, axons were visualized by immunohistochemistry (Fig. 2). When nerves were deviated under the dermal collagen layer, axon ends were found to be located far from the overlying epidermis (Fig. 1A). Furthermore, the epidermis did not contain axons from the deviated nerves (Fig. 2A). This finding also suggests that direct contact between the axons and epidermis was inhibited. Next, to create direct interaction between the nerves and epidermis without fibroblasts, nerves were placed on the dermal collagen layer after scratching the epidermal cells (Fig. 1C). This caused the epidermal cells to migrate and surround the rerouted nerves. The surrounding epidermis became thicker than the normal epidermis. The surrounding epidermis was largely dedifferentiated, as no Leydig cells, which are one type of

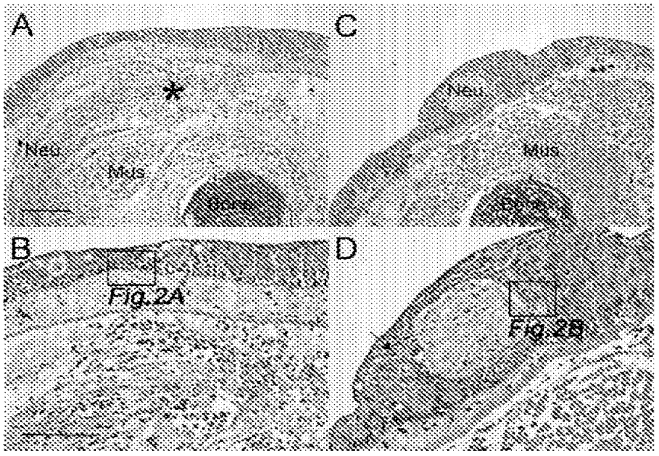


Fig. 1. Nerve deviation under or on the dermal collagen layer. **(A, B)** A nerve was deviated under the collagen layer. The nerve ends were seen under the dermal collagen layer (A, asterisk). Panel B is a higher magnification of panel A. **(C, D)** A nerve was deviated on the dermal collagen layer. The nerve was surrounded by the epidermis, which appeared to be undifferentiated. Panel (D) is a higher magnification of C. Sections were made 72 h after surgery and stained with Alcian blue and hematoxylin and eosin. Mus, muscle; Neu, neural tissues. Scale bar in (A) = 200 μm. Scale bar in C = 100 μm. (A) and (C) are the same magnification as (B) and (D). Arrow in (D) indicates a Leydig cell in the epidermis.

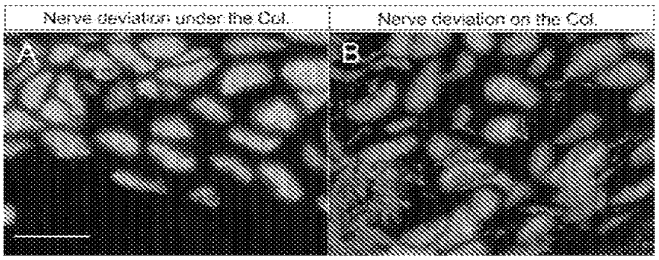


Fig. 2. Visualization of axon fibers. Axon fibers were stained with anti-acetylated tubulin antibody (red) and nuclei with DAPI (blue). **(A)** The deviated nerve under the dermal collagen layer did not reach the axons in the overlying epidermis. **(B)** Axon fibers could be seen in the epidermis. The deviated nerve extended its axons into the epidermis. A and B are the adjacent section of the boxed area in Fig. 1B and 1D, respectively. Scale bar in A = 50 μm. A and B are the same magnification.

secretory cell in axolotl skin (Fig. 1D, arrow), were present around nerves (Fig. 1D). In the surrounding epidermis, newly extended axon fibers could be seen (Fig. 2B). We then investigated *Sp-9* induction by the direct interaction between the nerves and epidermis. *Sp-9* was upregulated in samples in which nerves were deviated on the dermal collagen layer and was absent in normal skin (Fig. 3A). Consistent with histological observations, *Sp-9* upregulation was not detected in samples in which nerves were deviated under the collagen layer. Furthermore, we examined *Sp-9* expression by in situ hybridization (Fig. 3D, E) and detected *Sp-9* throughout the epidermis where axon fibers were detectable (Fig. 3B–D). Normal epidermis in the same section did not show intense signals for *Sp-9* expression (Fig. 3E). These results suggest that *Sp-9* expression is activated by the direct interaction between the nerves and epidermis, and AEC formation is induced by this direct interaction.

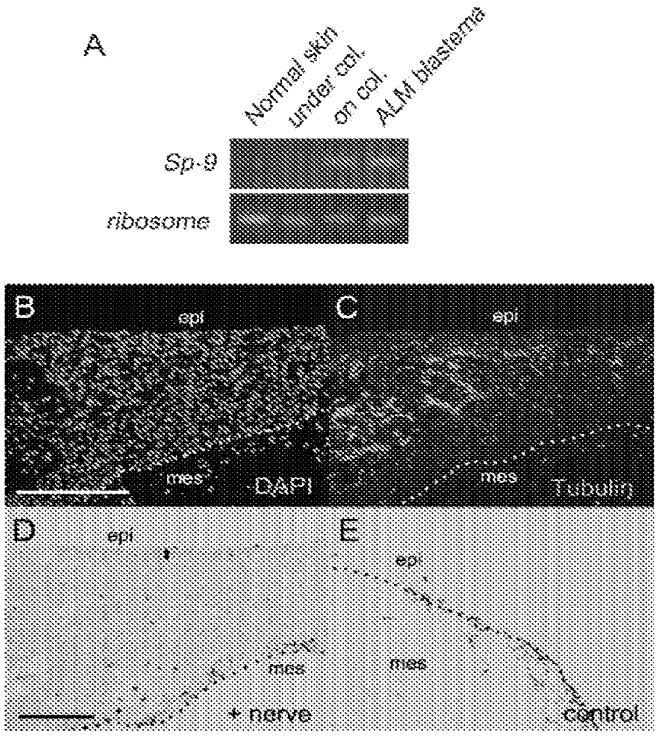


Fig. 3. *Sp-9* expression by nerve deviation. **(A)** RT-PCT analysis. *Sp-9* was not detected in normal skin. The rerouted nerve under the collagen layer (lane 2) did not activate *Sp-9* expression. In contrast, the deviated nerve on the collagen layer (lane 3) increased *Sp-9* expression. The ALM blastema also showed *Sp-9* expression. **(B–E)** Sections of the epidermis 7 days after nerve deviation. (B, C) Axons were visualized as green signals and nuclei as blue. Axons could be seen in the epidermis. (D) The *Sp-9* expression pattern was revealed by in situ hybridization shown in the adjacent sections of (B) and (C). (E) In the normal skin region of the same section as (D), no *Sp-9* signal was detected. Scale bar in B = 300 μm. Scale bar in D = 100 μm. (B) and (D) are the same magnification as (C) and (E). Dotted lines in (B–E) indicate the border of the epidermis. The upper side is apical, and the bottom side is basal. Epi. = epidermis. Mes. = mesenchyme (dermis and muscle).

Blastema induction rate decreased as wound healing progressed

The collagen layer in the dermis inhibited the direct interaction between the nerves and epidermis, resulting in the failure of AEC induction. In axolotl, collagen is reorganized during wound healing (Endo et al., 2004; Satoh et al., 2008a). We hypothesized that the AEC induction rate in ALM would decrease as collagen accumulated during skin wounding. To examine this, we created a wound, rerouted nerves on specific days after skin wounding, and investigated blastema formation. As expected, a blastema was seen after skin wounding each time the nerve was deviated (regular ALM surgery, data not shown). The blastema induc-

tion rate remained high when nerves were rerouted in 3-day-old wounds (Table 1, Fig. 4A, B); the wound healing region remained transparent three days after wounding (Fig. 4A). The blastema induction rate decreased in 5-day-old wounds (Table 1, Fig. 4C, D), and almost half of the examined limb grew a blastema (Table 1). At five days, the wound became slightly opaque (Fig. 4C), and at 10 days, the wound became more opaque and blastema induction was completely inhibited (Table 1, Fig. 4E, F). Pigment cells migrated into the 15-day-old wounds, suggesting that dermal tissue had regenerated (Fig. 4G) and that blastema induction was no longer possible (Table 1, Fig. 4G, H). We also investigated *Sp-9* expression by RT-PCR (Fig. 4I). *Sp-9* expression was consistently detectable in samples in which blastemas were formed. Very faint *Sp-9* expression was detectable in 10- and 15-day wounds in which nerves were deviated. We investigated collagen deposition by immunohistochemistry. Three- and 5-day-old wounds did not show much deposition (Fig. 5A–D), although we began to detect signals of type I collagen by day 5 (Fig. 5E–H). Collagen fibers were resynthesized in 10-day-old wounds (Fig. 5I–L), which was consistent with a previous study (Satoh et al., 2008a). Additional collagen fibers were deposited and organized in 15-day-old wounds (Fig. 5M–P). These results suggest that collagen deposition decreased the blastema induction rate.

Table 1. Blastema induction rate.

	Induction of ectopic blastema	No induction of ectopic blastema	Sum
Nerve deviation at day 0	10 (100%)	0 (0%)	10
Nerve deviation at day 3	11 (91.7%)	1 (8.3%)	12
Nerve deviation at day 5	13 (52%)	12 (48%)	25
Nerve deviation at day 10	0 (0%)	19 (100%)	19
Nerve deviation at day 15	0 (0%)	11 (100%)	11

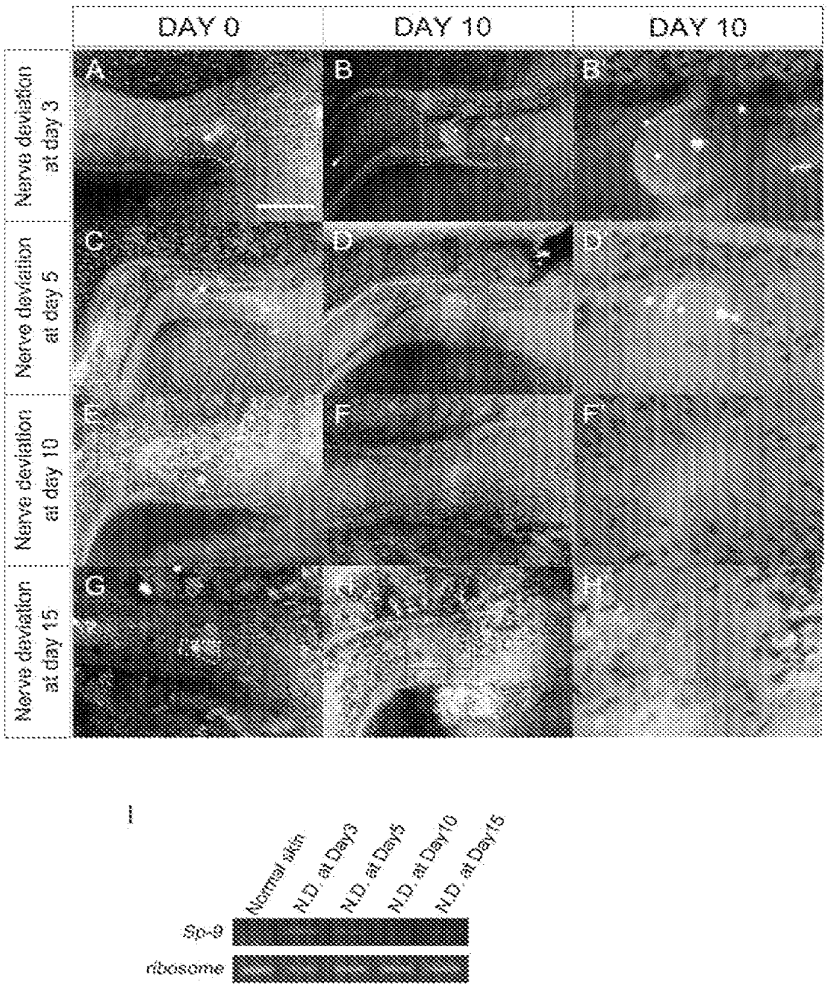


Fig. 4. Nerve deviation to wounds on specific days after wounding. **(A–H)** Anterior view of the limb with the rerouted nerve immediately (A, C, E, G) and 10 days (B, D, F, H) after surgery. Wound margin and putative wound margin were outlined by the dotted line in (A), (C), (E), and (G). Asterisks indicate the deviated nerve ends. (B′), (D′), (F′), and (H′) are the higher magnification views of the boxed region of (B), (D), (F), and (H), respectively. Arrows in B and D indicate the growing bump. All panels in (A–H) show heads to the right. Scale bar in A = 3 mm. (A–H) are the same magnification. **(I)** RT-PCR analysis showed that *Sp-9* was expressed. All samples were harvested 10 days after nerve deviation.

Placing an acellular collagen sheet on the deviated nerves inhibited blastema formation

To investigate whether collagen was related to the inhibition of blastema induction, we placed a collagen sheet on the deviated nerves at the same time as ALM surgery. An acellular collagen sheet was prepared as described in the Materials and Methods section. To confirm the absence of living cells, we prepared a section of the collagen sheet (Fig. 6A). The section was stained with hematoxylin to visualize the nuclei. However, nuclei were not detectable in this sheet, indicating only collagen bundles and no living cells. Next, a black axolotl-derived collagen sheet was labeled with green fluorescent dye and placed on the rerouted nerves (Fig. 6B). Ten days after surgery, no apparent blastema formation was observed (Fig. 6C). To confirm this, we cut sections (Fig. 6D–F) and found that the wound epithelium migrated over the collagen sheet (Fig. 6D). AEC was generally thickened; however, the wound epithelium that had migrated over the collagen layer was not thickened, suggesting no AEC formation. No axons were observed in the overlying epidermis (Fig. 6E). To further investigate this process, we performed in situ hybridization of the sections. *Prrx-1*, a blastema marker gene, was not detected (Fig. 6F), while the regular ALM blastema expressed *Prrx-1* in the absence of the collagen sheet (Fig. 6G). Next, we changed the nerve position in a similar experiment (Fig. 7). In this case, we rerouted the nerve on the grafted collagen sheet (Fig. 7A, B). Ten days after the surgery, the blastema

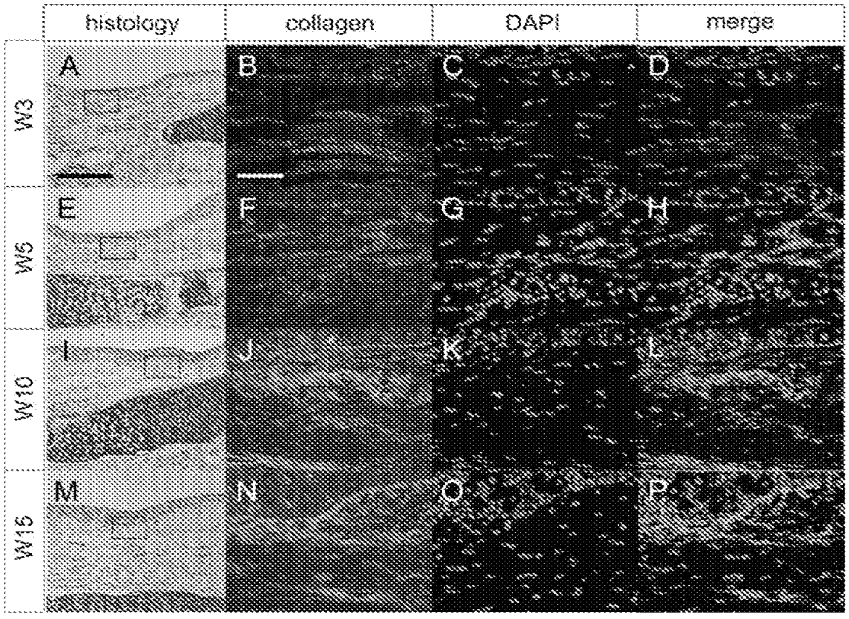
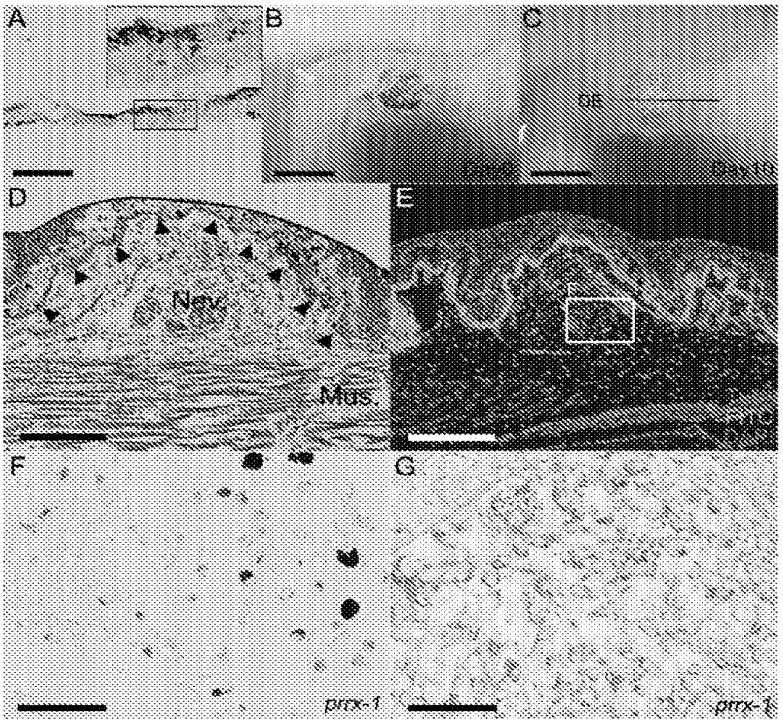


Fig. 5. Collagen reorganization during wound healing. **(A–D)** Three-day-old wound. **(E–H)** Five-day-old wound. **(I–L)** Ten-day-old wound. **(M–P)** Fifteen-day-old wound. (A, E, I, M) Alcian blue and hematoxylin and eosin staining. (B, F, J, N) Collagen was visualized by immunohistochemistry (green). (C, G, K, O) Nuclei stained with DAPI (blue). (D, H, L, P) Merged images. (A–D) Collagen deposition was not observed in the 3-day-old wound. B and C are higher magnification images of the boxed region in A. (E–H) Collagen synthesis was detected in the 5-day-old wound. (F–H) are higher magnification images of the boxed region in E. (I–P) Collagen bundles were observed late. (J–L) and (N–P) are higher magnification images of the boxed region in I and M, respectively. Scale bar in (A) = 0.7 mm. Scale bar in (B) = 100 μm. (A), (E), (I), and (M) are at the same magnification. The remaining images are of the same magnification as (B).



was observed (Fig. 7C, D). To confirm the identity of the blastema, sections were made. The grafted collagen was stained with green fluorescent dye as mentioned (Fig. 7E, G). The morphology of the collagen sheet was disrupted, suggesting that he grafted collagen sheet seemed to be partially digested by endogenous enzymes because a blastema shows higher MMP activities (Yang et al., 1999). *Prrx-1*-positive cells were accumulated between the collagen sheet and blastema epithelium (Fig. 7H, arrows). These data strongly suggest that blastema formation can be induced by a deviated nerve on a collagen sheet. Taken together with the inhibitory effect of a collagen sheet for blastema formation indicated in Fig. 6, our results suggest that collagen acts as a barrier for interaction between nerves and the epidermis.

DISCUSSION

Nerve function during AEC induction

Nerves are necessary for limb regeneration, and ALM provides many insights into nerve function during limb regeneration. Previous ALM studies have suggested that nerves play important roles in AEC induction, leading to blastema formation (Endo et al., 2004; Satoh et al., 2007; Satoh et al., 2008b; Satoh et al., 2009; Satoh et al., 2010a). FGFs are secreted from nerves during axolotl limb regeneration and may induce AEC formation (Satoh et al., 2008b). *Sp-9* is probably activated by nerve-secreted FGFs at the early phase of limb regeneration. In the case of limb development in higher vertebrates, *Sp-9* is also FGF-dependent (Kawakami et

Fig. 6. An acellular collagen sheet inhibited blastema induction. **(A)** A section of the prepared collagen sheet showing the absence of living cells. Insert shows higher magnification of the boxed region. **(B)** Green fluorescent-labeled collagen was placed on the deviated nerve. **(C)** Collagen sheet grafting did not result in blastema formation 10 days after surgery. **(D–F)** Sections of the collagen sheet-grafted and nerve-deviated wounds are indicated by the line in C. (D) The section was stained with hematoxylin and eosin. Arrowheads indicate the collagen sheet. Nev. = nerves. Mus. = muscles. (E) The collagen sheet was labeled with green fluorescent dye, and axons were visualized by immunohistochemistry (red). *Prrx-1* expression was investigated by in situ hybridization in the collagen sheet-grafted sample (F) and ALM blastema harvested 10 days after ALM surgery (G). Scale bar in B, D, and F are 2 mm, 500 μm, and 100 μm, respectively. E and G are the same magnification as D and F, respectively.

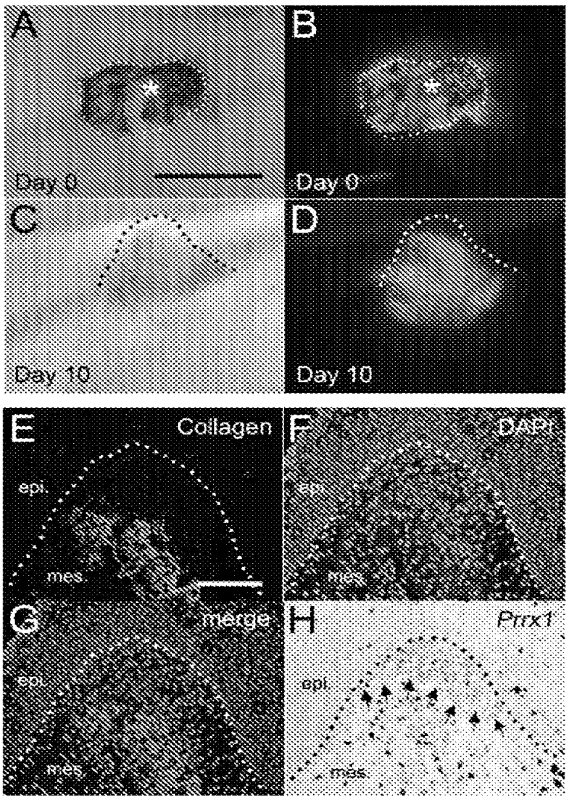


Fig. 7. Collagen sheet graft below the rerouted nerve did not inhibit blastema induction. Anterior view of the limb with the rerouted nerve immediately **(A, B)** and 10 days **(C, D)** after surgery. Asterisks in (A) and (B) indicate the end of the rerouted nerve. The grafted collagen was stained with green fluorescent dye as described in the Materials and Methods section. Dotted lines in (A) and (B) outline the wound margin. Dotted lines in (C) and (D) outline the induced bump. **(E–H)** Sections of the bump with collagen sheet. (E) The grafted collagen was detectable because it was stained with green fluorescent dye. (F) Nuclei were visualized by DAPI. (G) Merged image. Cells were accumulating between the grafted collagen sheet and the blastema epithelium. Such population was *Prrx-1*-positive (H, arrow). Epi. = blastema epithelium. Mes. = blastema mesenchyme. Scale bars in A and E are 2 mm and 400 μm, respectively. (A–D) are at the same magnification. (E–H) are at the same magnification.

al., 2004). This is not unexpected, as most limb regeneration processes are similar to those of limb development. The initial *Sp-9* expression pattern spreads throughout the wound epithelium and is then restricted to the basal layer (Satoh et al., 2008b; Fig. 3D). This finding suggests that the interaction of the blastema mesenchyme with the basal layer of the epidermis would be necessary for a restricted *Sp-9* expression pattern. Although a mesenchymal contribution may be necessary for a completely functional AEC, the nerves and epidermis are sufficient for initial *Sp-9* expression (Figs. 1–3). However, we believe that another issue remains unresolved. In our study, epidermal cells migrated, and therefore, we could not determine whether nonmigratory epidermal cells respond to neural factors. This question will be addressed in a future study.

Collagen deposition hinders blastema induction

The dermal collagen layer acts as a barrier for axon penetration (Figs. 4–6). However, a few axons were evident in the dermal layer. Hence, dermal collagen may not inhibit axon penetration completely but may make it difficult, which is consistent with the results shown in Figs. 4 and 5. It became difficult to induce a blastema due to collagen deposition (Table 1, Fig. 4). Deposition of collagen began to reconstitute dermal tissues after wounding without nerve deviation, a process that occurs during regular wound healing. Urodele amphibians can restore skin damage without scarring (Levesque et al., 2010), although collagen deposition occurs (Endo et al., 2004, Fig. 5). In higher vertebrates, collagen reconstitution occurs after skin wounding. Such reconstituted collagens, however, are not usually re-organized well, resulting in scar formation (Gurtner et al., 2008). The reason why urodele amphibians is able to undergo a scarless healing process has remained a mystery. To understand why, it would be helpful to describe collagen deposition processes in axolotl skin damage. Obvious collagen synthesis could not be detected in 3-day-old wounds (Fig. 5A–D). Epidermal cells maintain the ability to receive neural inputs at this time. However, 5-day-old wounds showed a decreased ability to form AEC (Table 1). Simultaneously, collagen deposition appeared to have just begun because faint collagen signals were observed by immunohistochemistry (Fig. 5E–H). Otherwise, epidermal cells begin losing their ability to receive neural inputs. I this case, the reason why epidermal cells start losing this ability needs to be determined. Finally, the blastema induction rate decreased to zero (Table 1, Fig. 5I–P), and collagen bundles were observed (Fig. 5J, N). Three possibilities account for the inhibition of blastema formation. First, reorganizing collagen acts as a physical barrier that blocks axon penetration. Because axons cannot penetrate through the collagen, the epidermal cells cannot activate *Sp-9* due to the lack of sufficient neural inputs. Second, as mentioned above, epidermal cells lose their ability to receive neural inputs. Third, reorganizing collagen absorbs neural factors, including FGFs. In fact, the extracellular matrix (ECM) traps FGF4 during chicken limb development (Schaller and Muneoka, 2001), and FGFs also bind well to ECM (Powers et al., 2000). Hence, newly synthesized collagen may absorb FGFs from nerves. Perhaps all of these factors are important for the early reactions during limb regeneration. A previous study showed that wound healing (collagen deposition) is inhibited on induction of AEC formation (Satoh et al., 2008a; Satoh et al., 2010a). Collagen deposition remains inhibited in a blastema environment. The blastema has many axon fibers, and the distal AEC is richly innervated (Satoh et al., 2008b; Thornton, 1956; Singer, 1949). Furthermore, the blastema is rich in FGFs secreted by AEC, nerves, and the blastema mesenchyme (Endo et al., 2000; Han et al., 2001; Yokoyama et al., 2001; Dungan et al., 2002; Satoh et al., 2008b). Thus, maintaining a lower level of collagen deposition may be important for the distal blastema environment. The opposite phenotype is observed between the blastema and wound healing during collagen deposition (dermal differentiation). Collagen function during limb regeneration should be studied more closely to allow a more detailed understanding of limb regeneration.

ACKNOWLEDGMENTS

This study was supported by the Naito Foundation and Special Coordination Funds for Promoting Sciences and Technology of the Ministry of Education, Sports, Culture, Science and Technology of Japan.

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(Received July 1, 2011 / Accepted September 27, 2011)

Skin Regeneration in Adult Axolotls: A Blueprint for Scar-Free Healing in Vertebrates

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Abstract

While considerable progress has been made towards understanding the complex processes and pathways that regulate human wound healing, regenerative medicine has been unable to develop therapies that coax the natural wound environment to heal scar-free. The inability to induce perfect skin regeneration stems partly from our limited understanding of how scar-free healing occurs in a natural setting. Here we have investigated the wound repair process in adult axolotls and demonstrate that they are capable of perfectly repairing full thickness excisional wounds made on the flank. In the context of mammalian wound repair, our findings reveal a substantial reduction in hemostasis, reduced neutrophil infiltration and a relatively long delay in production of new extracellular matrix (ECM) during scar-free healing. Additionally, we test the hypothesis that metamorphosis leads to scarring and instead show that terrestrial axolotls also heal scar-free, albeit at a slower rate. Analysis of newly forming dermal ECM suggests that low levels of fibronectin and high levels of tenascin-C promote regeneration in lieu of scarring. Lastly, a genetic analysis during wound healing comparing epidermis between aquatic and terrestrial axolotls suggests that matrix metalloproteinases may regulate the fibrotic response. Our findings outline a blueprint to understand the cellular and molecular mechanisms coordinating scar-free healing that will be useful towards elucidating new regenerative therapies targeting fibrosis and wound repair.

Citation: Seifert AW, Monaghan JR, Voss SR, Maden M (2012) Skin Regeneration in Adult Axolotls: A Blueprint for Scar-Free Healing in Vertebrates. PLoS ONE 7(4): e32875. doi:10.1371/journal.pone.0032875

Editor: Zhongjun Zhou, The University of Hong Kong, Hong Kong

Received September 15, 2011; **Accepted** February 3, 2012; **Published** April 2, 2012

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Funding: The Ambystoma Affymetrix Genechips were designed from a transcript assembly funded by NIH-NCRR (R24-RR016344). Most of the transcripts were generated under the NIH-NCRR grant and an Army Research Office grant (MURI: W911NF-09-1-0305) to Ken Muneoka, David Gardiner, and SRV. Additional transcripts were provided by Elly Tanaka through funding from the DFG Center for Regenerative Therapies, and Bernd Fritsch through funding from NIH-NIDCD (R01-DC005590-07S1). The axolotls were obtained from the Ambystoma Genetic Stock Center at the University of Kentucky, which is funded by the National Science Foundation (DBI-0951484). This study was supported by NIH 5RC2NS069480 to MM and AWS is funded by NIH-NIDDK 5T32DK074367. Publication of this article was funded in part by the University of Florida Open-Access Publishing Fund. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Among its many functions, the skin is primarily responsible for maintaining the structural and physiological barrier between an organism's internal and external environment. As the first line of defense against external insult the skin is injured more frequently than any other tissue and resulting damage, while repairable, leads to permanent scarring in mammals [1]. At least 100 million people in the developed world acquire scars each year in response to trauma and surgery and the result is a spectrum of pathologies from thin line surgical scars to hypertrophic and chronic non-healing wounds [2,3,4]. Adding to this medical burden, burn injuries, which often elicit an over-exuberant fibrotic response, result in hypertrophic scarring with treatment costs in the US alone accounting for $4 billion annually [5]. While not as complex as regenerating a human digit or limb, the ability to develop regenerative strategies that lead to scar-free healing in adult skin remain tantalizingly out of reach. Understanding how to coax the natural wound repair process towards a regenerative outcome remains the grail of wound healing research.

Our knowledge of the molecular and cellular events during mammalian tissue repair is extensive (see refs [1,6,7,8]) and yet, even with such broad understanding of the wound repair process, regenerative medicine has failed to develop therapies that can perfectly regenerate skin. This stems partly from the dynamic reciprocity of cellular interactions and signaling pathways and partly from a lack of appropriate models to observe these interactions in a regenerative environment [9]. While wound repair in fetal mammals [10,11,12,13,14] and marsupials [15] has provided insight into the cellular and molecular regulation of scar-free healing, comparisons of wound repair between fetal mammals and adults has limitations, both biological and practical [16]. The developing fetus, at the time when it heals scar-free, has an immature endocrine system, is immuno-incompetent, is contained in a moist sterile environment, and its cells are in a state of chronic hypoxia [16]. Adult skin is more completely differentiated and adult wounds are open to desiccation and infection, two factors that seriously complicate wound repair. Other promising models of scar-free healing, such as the MRL mouse, which share the ability to regenerate ear punches with rabbits, hares, pikas, cows, pigs and cats [17,18,19] has proven less than perfect when challenged to heal excisional skin wounds [20,21] casting doubt on the special regenerative powers of this inbred mouse model.

Compared to other vertebrates, urodeles possess the amazing capacity to regenerate their limbs, hearts, lenses, spinal cords, tails, internal organs and joints. Observations from studies examining

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limb regeneration have been extrapolated to the skin, but direct comparisons to the processes of cutaneous wound repair have rarely been made [22]. Studies examining limb wounds have yielded insight into the process of re-epithelialization [23,24,25,26] and regeneration of the basement membrane [27,28], but the dynamics of dermal regeneration have remained obscure. Direct study of wound repair in urodele skin outside of regeneration fields like the limb and tail, however, has not been undertaken.

Given their seemingly absolute powers of regeneration, a recurring question has been whether wounds made outside of regenerating structures (e.g. limbs and tails) in adult urodeles are capable of scar-free healing or, like adult anurans, heal with a scar [29]. In this study we examined full thickness excisional (FTE) wound healing of dorsal back skin in adult axolotls. Using an established mammalian excisional wound model to directly characterize cutaneous wound healing in adult axolotls, we examined hemostasis, inflammation, new tissue formation and remodeling processes. Additionally, we induced metamorphosis in adult axolotls to test the hypothesis that loss of larval skin characters and transition to a terrestrial form results in fibrotic scarring following FTE flank wounds. Here we demonstrate that both aquatic and terrestrial axolotls are capable of perfect, scar-free skin regeneration. We discuss these findings in the context of mammalian wound repair and present a blueprint for investigating the cellular and molecular mechanisms that regulate scar-free skin healing in adult vertebrates.

Results

Full Thickness Excisional Flank Wounds are Perfectly Regenerated in Adult Axolotls

Adult axolotls (*Ambystoma mexicanum*) are aquatic and paedomorphic, exhibiting several juvenile features as adults (e.g. retention of leydig cells, pseudo-stratified epidermis, external gills). Although the dermis of adult axolotl skin is typical for amphibians, the epidermis is pseudo-stratified and lacks a well-defined stratum corneum (Figure 1A, B). Above the stratum germinativum, epithelial cells are interspersed with leydig cells (specialized cells containing highly granulated cytoplasm) that are characteristic of larval amphibian skin (Figure 1B and [30,31]). The dermis contains epidermally-derived mucous and granular glands that are embedded within the stratum spongiosum, a loose network of thin collagen fibers and fibroblasts that lies above the stratum compactum (Figure 1A, B and Figure S1A, B). The stratum compactum forms a thickened sheet of compressed collagen fibers that sits atop hypodermis and separates the skin from the underlying muscle (Figure 1A).

It has been reported that wounds made outside of regeneration fields (i.e. limbs, tail, head) heal with a scar [32] and to date, this controversy remains unresolved. To directly test the hypothesis that axolotls can perfectly regenerate (i.e. heal scar-free) full thickness excisional (FTE) wounds, we made circular 4mm FTE wounds through the dermis into the dorsal flank muscle (n = 12) and observed the wound healing process over 180 days (Figure 1C-H, Figure 2, Figures S2). In response to injury blood flowed into the wound bed, clotted, but did not form a scab (Figure 2). Epithelial cells from the wound margins migrated across the underlying muscle and accumulated plasma and completely re-epithelialized the wound within 24hrs (Figure 1C, green arrows indicate plasma). Following re-epithelialization numerous blood cells (leukocytes and erythrocytes) were apparent in the wound bed and the neoepidermis remained in close proximity with the underlying muscle and plasma over the next 7–10 days (Figure 1D). Fourteen days post injury we observed dermal fibroblasts in the wound bed and Masson's Trichrome staining revealed newly deposited extracellular matrix (ECM) (Figure 1E). Twenty-one days post injury muscle fibers continued to fragment liberating mono-nucleate cells into the surrounding tissue and a robust ECM formed between the epidermis and muscle (Figure 1E, F). Forty-seven days post-injury new epidermal organs were present in the regenerated stratum spongiosum (Figure 1G and Figure S2A). The stratum compactum, however, had not completely regenerated and this was clearly evident at its margins (Figure 1G and Figure S2B). Although the timing to regeneration varied among individuals, full thickness skin, including epidermal organs and underlying muscle, was completely regenerated by 80 days (Figure 1H and Figure 2). Maturation and development of glands and the stratum compactum continued over the next 100 days (Figure S2C).

New ECM Deposition Corresponds with Formation of the Lamina Densa

Previous work in regenerating newt limbs suggested that reformation of the basement membrane (BM) facilitates dermal regeneration and its delayed formation permits blastema formation [28]. We followed BM regeneration after re-epithelialization and asked whether it occurred prior to the onset of dermal regeneration in excisional flank wounds. In uninjured skin the BM is visible as a thick fibrous band separating epidermis from dermis and is continuous except where mucous glands interject into the epidermis (Figure 3A). Following re-epithelialization histological staining revealed a thin, immature structure beneath the new epidermis (Figure 3A). The BM continued to mature and was completely regenerated at least 47 days after wounding (Figure 3A; yellow arrows D47). Interestingly, complete regeneration of the BM corresponded to regeneration of the dermis (except for stratum spongiosum) (Figure 3A and Figure 1G).

In order to test if assembly and maturation of the BM corresponded to the onset of ECM deposition we analyzed formation of the lamina lucida and lamina densa using antibodies that recognize laminin (lamina lucida) and collagen type IV (lamina densa) (Figure 3B). Both proteins were detectable in the BM of uninjured skin, and surrounding glands and muscle fibers (Figure 3B). Following re-epithelialization, basal epidermal cells were negative for both laminin and collagen IV (Figure 3B; white arrows). We detected strong and continuous laminin staining beneath the epidermis 7 days post injury indicating lamina lucida reformation (Figure 3B). Collagen IV was not detected continuously beneath the epidermis until D14 (corresponding to the onset of ECM deposition) (Figure 3B).

In addition to protein localization we tracked epidermal gene expression of the BM components *collagen type IV* and *laminin alpha* 1 (*Lama1*), *laminin beta* 1 (*Lamb1*), *laminin gamma* 1 (*Lamc1*), which together form the protein laminin-111 (Table S1). Expression of all three laminin-111 subunits was unchanged during re-epithelialization (Table S1). Expression of both *alpha-1* and *beta-1* components of laminin-111 increased 5.6-fold and 4.1-fold respectively between D1 and D3, and continued increasing at D7 post injury (Table S1). In the skin basement membrane of humans and mice, two hetero-trimeric collagen type IV molecules consisting of alpha1(IV)$_2$/alpha2(IV) and alpha5(IV)$_2$/alpha6(IV) chains exist [33]. We examined expression of each of these alpha chains following injury and found that expression of *alpha1* and *alpha2* chains were down regulated following injury, remained below baseline levels until D3, after which they returned to baseline levels at D7 post injury (Table S1). Whereas expression of the *alpha6* chain remained unchanged, the *alpha5* chain was upregulated 4.9-fold at D1 and sustained through D7 (Table S1).

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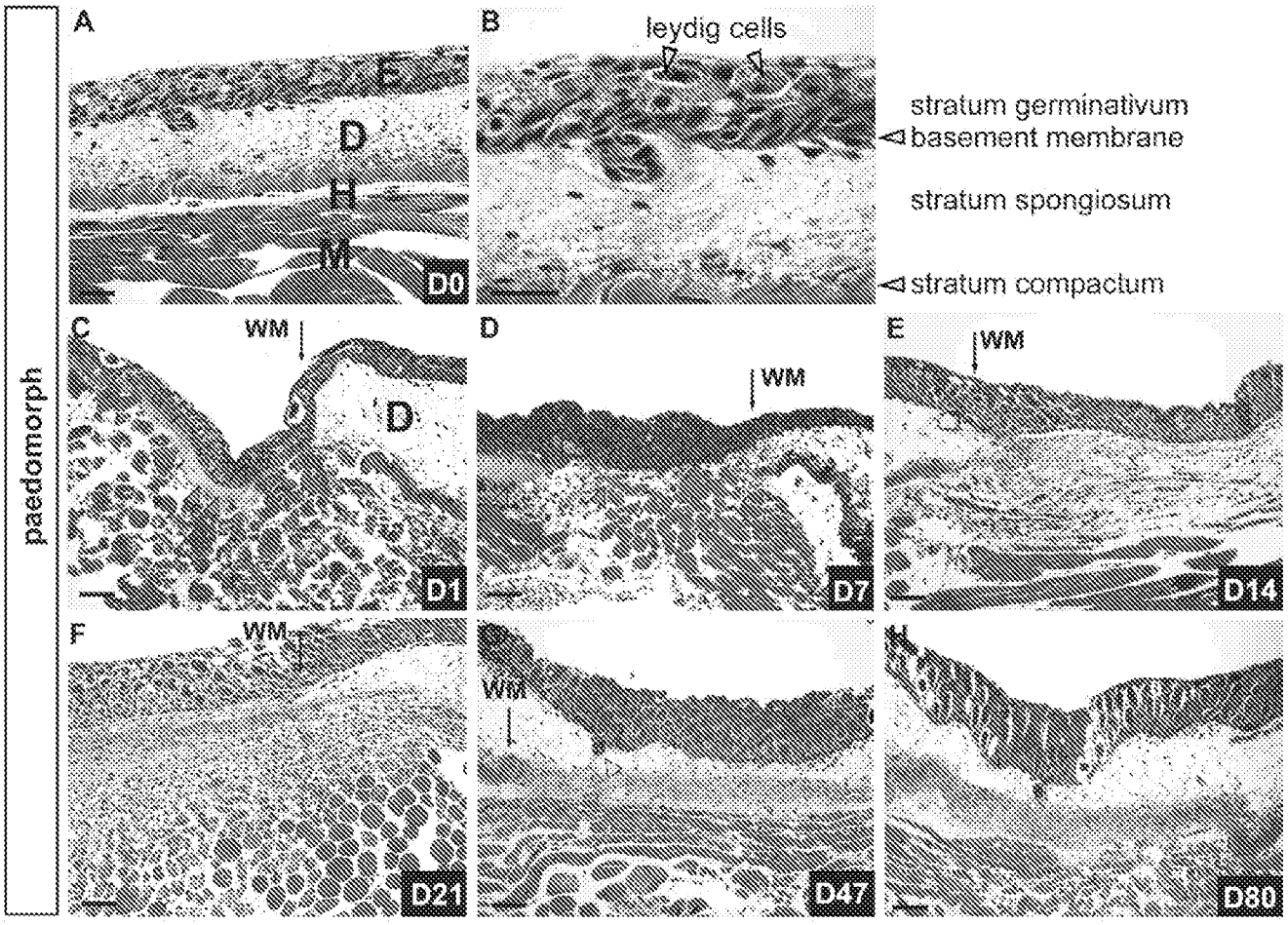


Figure 1. Scar-free healing of full thickness excisional wounds in adult axolotls. A) Masson's trichrome staining of uninjured dorsal flank skin in axolotls showing epidermis (E), dermis (D), hypodermis (H) and underlying muscle (M). B) Magnified image of epidermis and dermis. The epidermis is pseudo-stratified and contains epithelial cells and leydig cells (yellow arrows) while the dermis is divided into the stratum spongiosum (containing glands and dermal fibroblasts) atop the densely compacted ECM of the stratum compactum. C-H) Scar-free healing over 80 day period following full thickness excisional wounding. C) One day post injury (dpi) the wound bed is completely re-epithelialized. Some blood plasma has accumulated beneath the neoepidermis (green arrows) (wound margin = WM). D) Seven dpi there is little evidence of a fibrin clot between the epidermis and underlying muscle and no new ECM has been deposited. Green arrows depict residual blood plasma. E) Fourteen dpi fibroblasts are visible beneath the epidermis where new ECM is deposited (blue staining) and muscle cells are fragmenting into individual myoblasts. F) Twenty-one dpi a thick band of transitional matrix is visible beneath the epidermis. Collagen is visible within the regenerating muscle. G) Forty-seven dpi the underlying muscle has completely regenerated and is devoid of collagen. Skin glands (yellow arrow) have regenerated and descended from the epidermis. The dermal stratum spongiosum has reformed and the stratum compactum is coalescing. H) Eighty dpi all skin layers have regenerated. Scale bars = 100 μm.
doi:10.1371/journal.pone.0032875.g001

Taken together, these results demonstrate that regeneration of the BM proceeds through formation of the lamina lucida followed by production of the lamina densa and that complete lamina densa formation corresponds to the onset of ECM production in the wound bed.

Metamorphic Axolotls Exhibit a Delay in Skin Regeneration Compared to Paedomorphic Axolotls

While adult mammals are incapable of regenerating full thickness skin wounds, fetal mammals exhibit scarless healing of similar type wounds [12]. Similarly, while pre-metamorphic anurans heal scar-free, post-metamorphic anurans have been documented to heal flank wounds through scar formation [29]. Adult axolotls retain several larval skin features (e.g. leydig cells, pseudo-stratified epithelium), thus we asked if these characteristics facilitate their ability to heal wounds scar-free. To test this hypothesis we exploited the fact that normally aquatic axolotls retain the ability to undergo metamorphosis to a terrestrial form through administration of thyroxine and we induced metamorphosis in adult axolotls (controlling for age and size with sibling paedomorphs). Comparing uninjured epidermis between both forms we noted two major differences; first, granular glands that occupied relatively little space in the paedomorph dermis were greatly enlarged and occupied most of the stratum spongiosum while mucous glands appeared similar in form between morphs (Figure 4A and Figure S3A). Second, the epidermis no longer contained leydig cells and had transitioned to a completely stratified epithelium exhibiting a well-defined stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum (Figure 4B).

We next examined wound repair in metamorphs following 4mm full thickness excisional wounds over 180 days (Figure 4C-H

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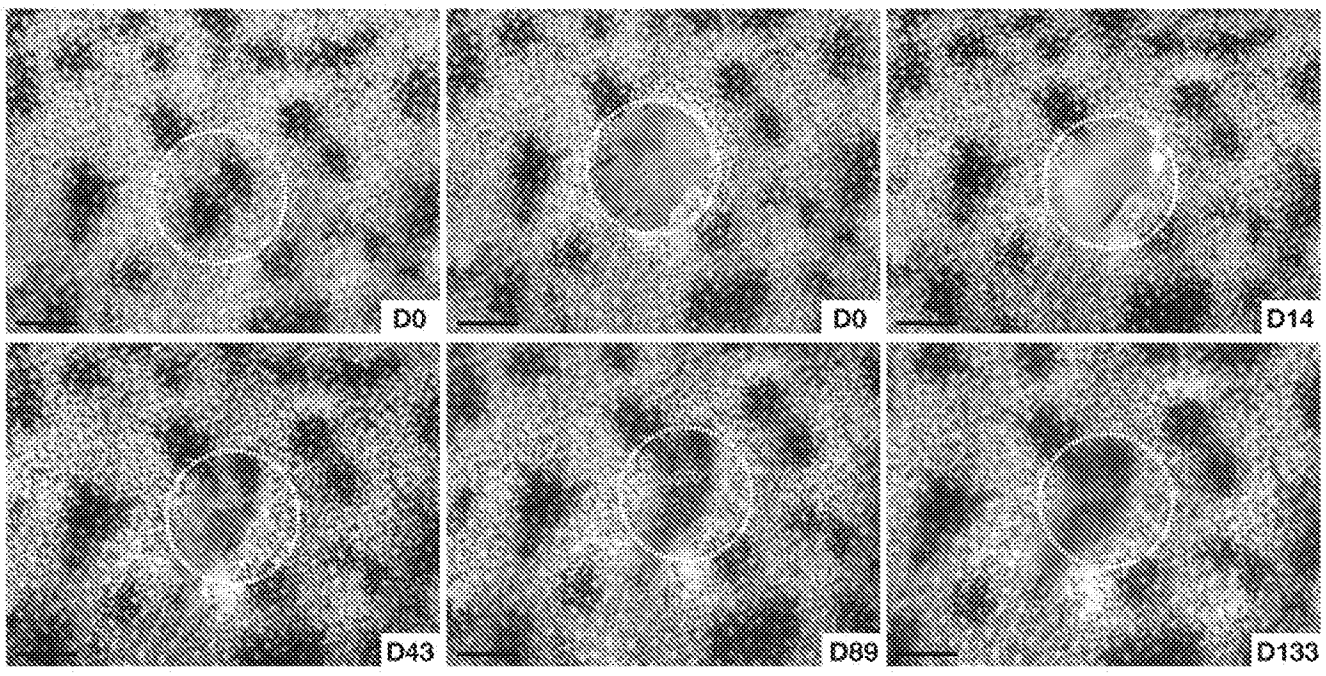


Figure 2. Scar-free healing in adult axolotls. Regeneration of dorsal flank skin following 4 mm full thickness biopsy punch wounding. White circles depict area of original injury. Individual pigment cells are visible at D14 in the overlying epithelium. Regeneration and scar-free healing at D89. Contraction is evident at the wound edges after D14. Scale bars = 2.0 mm.
doi:10.1371/journal.pone.0032875.g002

and Figure S3B-E). Twenty-four hours post injury re-epithelialization was not complete and the leading edge of migrating epidermal cells was visible both macroscopically and in section (Figure 4C). Re-epithelialization was complete by 72hours post injury and the epidermis had re-stratified to establish apical/basal polarity along the regenerating basement membrane (Figure 4D). Seven days post injury the wound bed contained plasma beneath the epidermis and within the fragmented muscle, along with large numbers of erythrocytes and leukocytes (Figure 4D). ECM deposition began as it had in the paedomorph approximately 10–14 days post injury and we noted that it appeared to extend deep into the muscle (Figure 4E). Twenty-one days post injury the ECM was dense within the wound bed, new vasculature was present in both the epidermis and ECM, muscle fibers continued to fragment and aggregations of cells appeared in the epidermis suggesting glands were beginning to regenerate (Figure 4F and Figure S3B).

Complete dermal regeneration was delayed in metamorphs (compare Figure 1G and Figure 4G). While epidermal organs regenerated in both forms after 40 days, the wound bed and underlying muscle still contained densely compacted extracellular matrix in metamorphs (Figure 4G and Figure S3C). After 80+ days the stratum spongiosum had regenerated but the stratum compactum remained incomplete (Figure 4H). After 120 days, the wound site resembled an 80-day regenerating wound in paedomorphs and a few collagen deposits still persisted in the underlying muscle (Figure S3D). Fibrosis was not resolved until at least 148 days and while mucous glands regenerated to pre-wound size, granular glands remained small even after 148 days (Figure S3E). Taken together these findings suggest that flank skin in adult metamorphic axolotls can completely regenerate following FTE wounding, but the time required to regenerate both the stratum compactum and mature granular glands is lengthened compared to paedomorphs.

The Rate of Re-epithelialization is Slower in Metamorphs

Following injury, re-epithelialization of the wound bed is necessary to re-establish epidermal integrity. In various rodent species, untreated FTE wounds re-epithelialize between 3 and 7 days (personal obser.). In contrast, aquatic axolotls re-epithelialize 4mm flank wounds in ~18hrs (Figure 1C and Figure S4). We compared the rate of re-epithelialization between paedomorphs and metamorphs to test whether the completely stratified epidermis of terrestrial axolotls affected its ability to cover the wound bed. Using an antibody to a wide-spectrum of cytokeratins we labeled the epidermis 24 and 72hrs after injury (Figure 5). Compared to the completely re-epithelialized wound bed of paedomorphs, wound edge epithelial cells in metamorphs had just begun migrating 24 hrs after injury (Figure 5A, B). Metamorph re-epithelialization was complete by 72 hrs (Figure 5C). Examination of the wound epidermis in paedomorphs showed leydig cells present in the new epidermis (Figure 5D). The leading edge of metamorph epidermis appeared pseudo-stratified (reminiscent of paedomorph epidermis without leydig cells) and moved as a sheet of cells with a single cell at the leading edge (Figure 5E). Following re-epithelialization, the wound epidermis re-established a stratified epithelial morphology (Figure 5F). In conjunction with this delay in re-epithelialization we observed a concomitant delay in appearance of the BM components laminin and collagen type IV reflecting the dependency of BM reassembly on the timing of complete re-epithelialization (data not shown). We examined the expression of the same molecular components of laminin and collagen type IV as we did for paedomorphs and did not detect a significant increase in expression prior to D7 (Table S1). These results show that stratified epidermis in metamorphs requires an extended activation period (relative to paedomorphs) before migration begins and this delay contributes to a slower rate of re-epithelialization.

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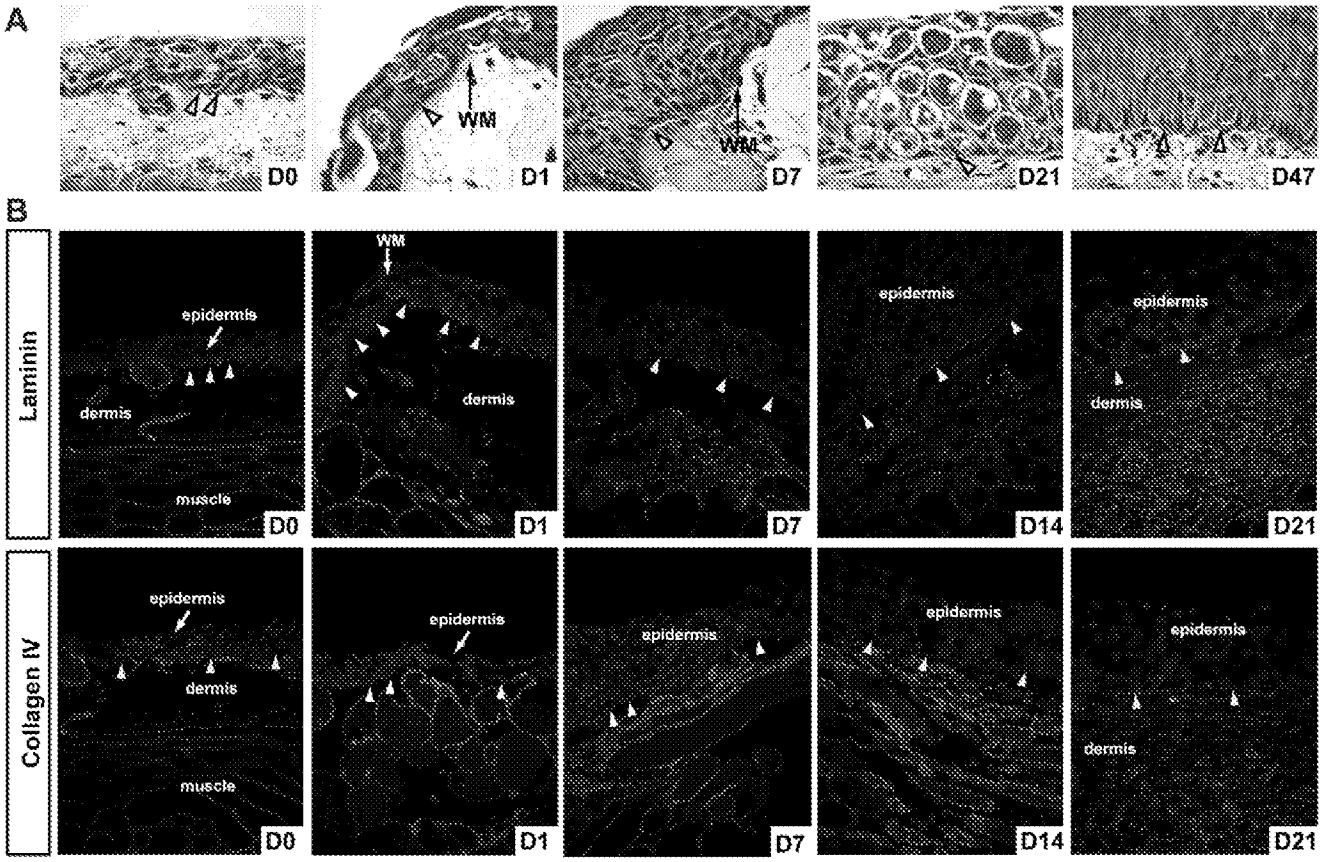


Figure 3. Lamina lucida and lamina densa regenerate before new ECM deposition. A) Histological examination of basement membrane (BM) regeneration in axolotls. The uninjured BM is visible as a thick blue-stained fibrous band (yellow arrows). An immature BM has begun to reform (yellow arrow D1) after re-epithelialization and is visible at the wound margin (WM) in contrast to the uninjured BM. The regenerated BM is visible at D47. Yellow arrows at D7 and D21 indicate reforming BM. B) Examination of lamina lucida (laminin) and lamina densa (collagen type IV) during basement membrane regeneration. The uninjured BM is positive for laminin and collagen type IV (yellow arrows) as are the basement membranes surrounding glands and muscle fibers. Following re-epithelialization the basal lamina of the epidermis is negative for laminin and collagen type IV (white arrows) and this is clearly evident at the wound margin (WM). Seven days post injury the BM stains strongly for laminin indicating reformation of the lamina lucida, while staining for collagen type IV is punctuated. The lamina densa is regenerated by D14 based on continuous collagen type IV staining and persists during dermal regeneration.
doi:10.1371/journal.pone.0032875.g003

Migrating Keratinocytes and Neoepidermis Express High MMP Levels

Epidermal expression and enzymatic activity of serine proteinases and matrix metalloproteinases has been shown to affect keratinocyte migration and ECM degradation during the early stages of mammalian wound healing [1]. In order to begin addressing the role these enzymes play during skin regeneration in axolotls we analyzed the expression of the serine proteinase *plasminogen tissue activator* (*PLAT*), matrix metalloproteinase (*MMP*) family members and the *tissue inhibitors of metalloproteinases* (*TIMP 1–4*) in migrating and neoepidermis following excisional wounding at D1, D3 and D7 in paedomorphs and metamorphs (Table S1). *PLAT* functions to convert plasminogen to plasmin and consistent with the absence of a fibrin clot was not expressed either in uninjured axolotl skin or following injury in the epidermis (Table S1).

During skin regeneration in axolotls we found a strong *MMP* response to injury in both migrating keratinocytes and neoepidermis in paedomorphs and metamorphs (Table S1). For all *MMPs* demonstrating significant changes in gene expression following injury, the expression kinetics generally followed two patterns; (1) a strong upregulation at D1 followed by an equally strong decrease at D3 and a continued decrease or leveling off at D7 and (2) a strong upregulation at D1 which remained higher than baseline at D3 and then was downregulated at D7 (Figure 6).

The collagenases *MMP1* and *axCOL* (an amphibian specific collagenase orthologous to newt *nCOL*) followed the first pattern with *MMP1* exhibiting a particularly strong response, increasing 425-fold (paedomorphs) and 502-fold (metamorphs) at D1, while remaining 88-fold (paedomorphs) and 61-fold elevated (metamorphs) at D7 (Table S1). The stromelysin *MMP3/10a* exhibited a similar expression profile as the collegenases except D7 levels returned near baseline (Figure 6). *MMP3/10b* levels, while exhibiting strong upregulation at D1, exhibited a slow decreasing rate of expression (pattern 2) compared to *MMP3/10a* and remained elevated at D7 (Figure 6). While *MMP19* and *MMP28* followed expression pattern 1 for paedomorphs, expression of both genes remained high in metamorphs mirroring the lag time in the metamorphic re-epithelialization rate (Figure 6). The gelatinase *MMP9* exhibited pattern 1 expression kinetics and responded 2-fold greater in metamorphs. *MMP2* was the one exception to the generally observed patterns. *MMP2* was effectively turned off at D1, remained off at D3 and then was upregulated in both paedomorphs and metamorphs (Figure 6). Given the strong

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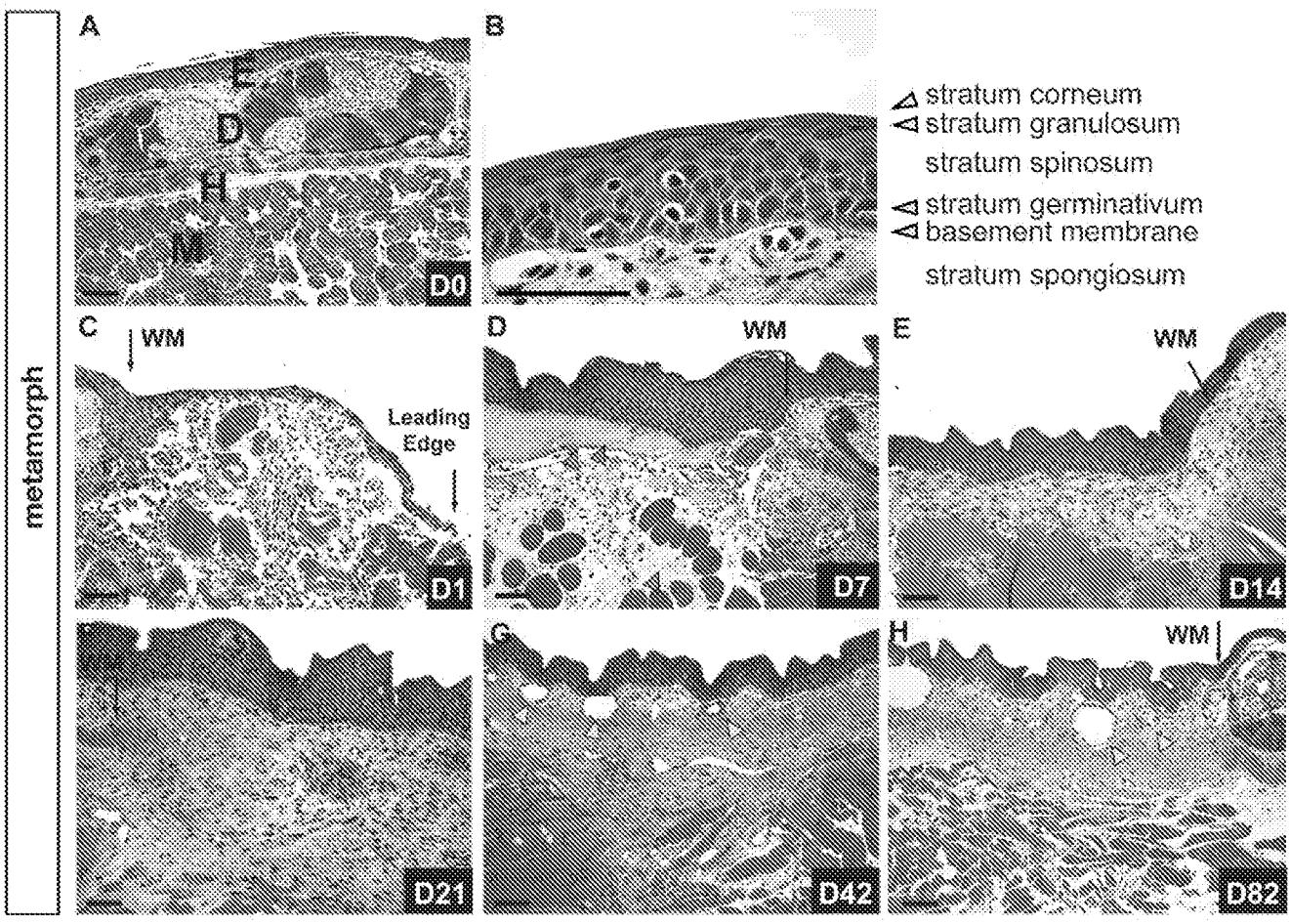


Figure 4. Metamorphic axolotl skin heals scar-free but slower compared to paedomorphs. A–B) Morphology of uninjured axolotl skin after metamorphosis. A) Masson's trichrome staining showing epidermis (E), dermis (D) containing enlarged granular glands in stratum spongiosum atop stratum compactum, hypodermis (H) and muscle (M). B) High magnification of epidermis showing stratified epithelium. Leydig cells have disappeared and the epidermis now contains a well-defined stratum spinosum, granulosum and corneum. C-H) Wound healing following FTE wounds over 82-day period. C) One day post injury (dpi) epithelial cells have begun to migrate but the wound is not re-epithelialized. Erythrocytes are visible in the wound bed and between muscle fibers undergoing histolysis. D) Re-epithelialization is complete 3 dpi and at 7 dpi coagulated plasma (green arrows), erythrocytes and leukocytes are visible in the wound bed. E) Fourteen dpi fibroblasts are visible in the wound bed and new ECM is deposited (blue staining). The wound margin (WM) is still clearly visible. F) The wound bed 21 dpi is rich in ECM. This ECM extends deep into the underlying muscle fibers which are fragmenting into myoblasts. G). Regenerating glands (yellow arrows) are present in the dermis 42 dpi and the stratum spongiosum is beginning to develop. The underlying muscle continues to remain damaged with deep pockets of collagen persisting beneath the wound. H) Eighty dpi the dermis is partially regenerated but the stratum compactum has not coalesced. Some collagen still persists deep in the muscle and both mucous and granular glands have regenerated (yellow arrows). Scale bars = 100 μm.
doi:10.1371/journal.pone.0032875.g004

response to injury reflected in *MMP* levels, we also analyzed expression levels for the *tissue inhibitors of MMPs* (*TIMPs*) (Table S1). Only *TIMP1* changed significantly, with paedomorphs exhibiting pattern 1 expression and metamorphs exhibiting pattern 2. Interestingly, axolotls appear to have two copies of *TIMP1* based on sequence analysis and this second copy did not change in either morph following injury (data not shown). Previous experiments during newt limb regeneration have shown that *MMP* transcription correlates well with their enzymatic activity [34] and the high sequence similarity between newt and axolotl *MMP* sequences strongly supports MMP orthology. We conducted gelatin zymography during paedomorphic tail regeneration and found that transcription and activity of MMP9 were well correlated, thus supporting this association in axolotl (Figure S5 A-C). This confirms recent work during axolotl limb regeneration demonstrating the activity of axolotl MMP [35]. Taken together these results suggest MMPs may play a key role during keratinocyte migration and regeneration, and suggest that sustained activity may control the timing of ECM deposition.

Metamorphic Axolotls Exhibit Increased Leukocyte Infiltration

During wound repair, adult mammals exhibit a stereotypical inflammatory response characterized by infiltration and subsequent removal of leukocytes [36]. The trade-off between regeneration and fibrosis has been postulated to result from a weak inflammatory response with greater inflammation contributing to increased fibrosis [37]. In order to characterize the inflammatory response during skin regeneration, we assayed the wound bed for L-plastin positive cells, a pan-leukocytic marker conserved in vertebrates. To control for individual variation we harvested wound tissue from the same animal (4 wounds per animal) at D0, D1, D3, D7 and D14 from paedomorphs (n = 4)

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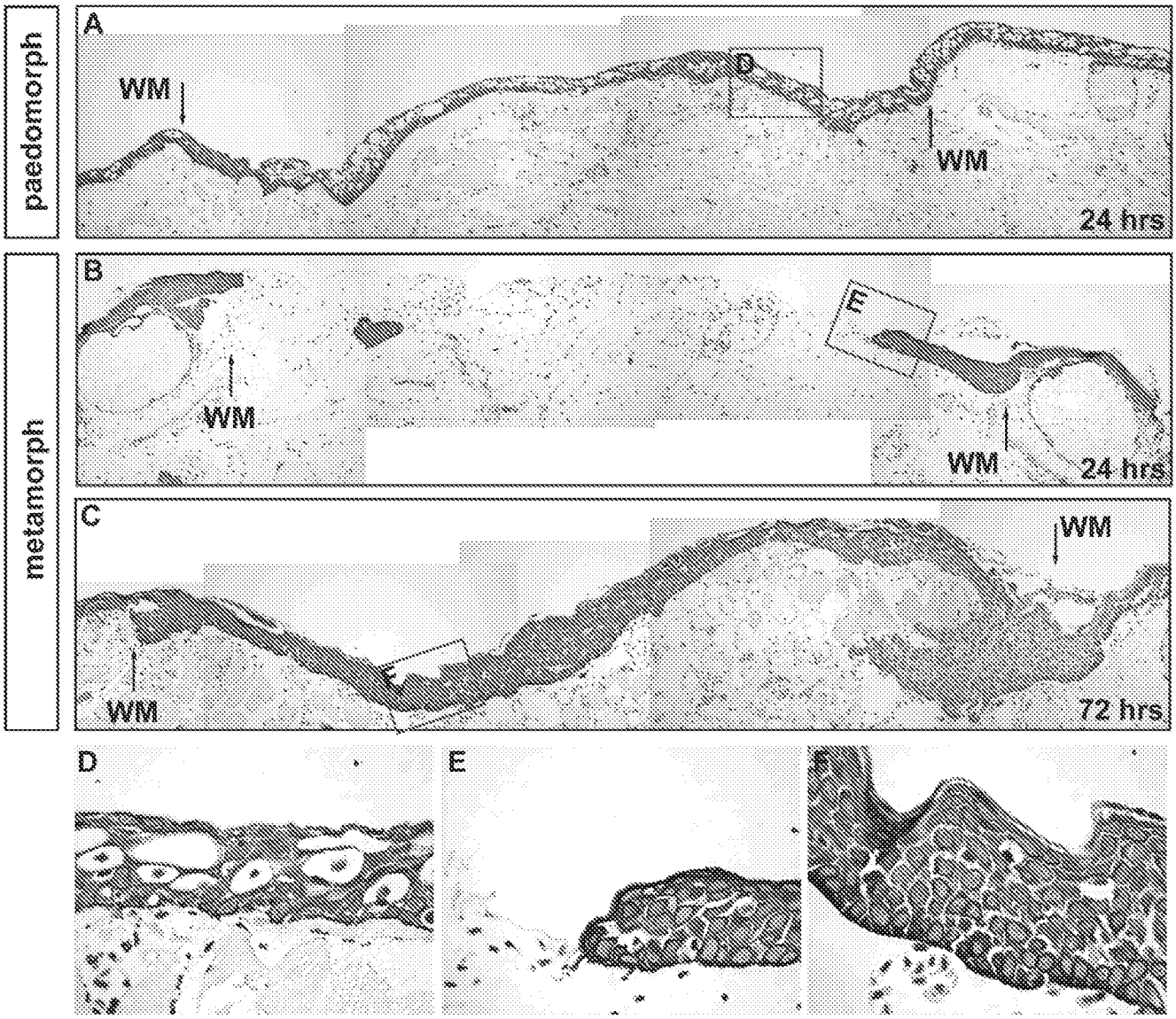


Figure 5. The rate of re-epithelialization is slower in metamorphs compared to paedomorphs. Epidermis was labeled with a pan-cytokeratin antibody. A) The wound bed is completely re-epithelialized 24 hrs after injury in paedomorphs. The wound margins (WM) are visible where the stratum compactum is disrupted. B, C) Wound edge keratinocytes have just begun migrating 24 hrs dpi and re-epithelialization is complete by 72 hrs dpi in metamorphs. D) Leydig cells are present in the paedomorph neoepidermis. E) The leading edge of migrating metamorph keratinocytes. The epidermal cells appear to move as a group of cells with one cell at the leading edge. F) After re-epithelialization is complete in metamorphs the epidermis becomes re-stratified.
doi:10.1371/journal.pone.0032875.g005

and metamorphs (n = 4). Examining the number of L-plastin positive cells we found both morphs exhibited a robust infiltration of leukocytes into the wound bed (Figure 7A and Figure S6A). We used a 2-way ANOVA to examine the effects of morph (metamorph vs paedomorph) and day post injury on total leukocyte numbers (with individual as a random effect to allow for repeated sampling). This analysis yielded a small p-value for the effect of morph ($F = 3.05$, $p = 0.095$). An examination of each individual over time showed that one paedomorph exhibited much higher levels of L-plastin positive cells (suggesting chronic stress or sickness) compared with the other three individuals (Figure S6B, C; paed 4). Therefore we analyzed the data without this individual and found that metamorphs had a significantly higher number of leukocytes following injury ($F = 5.32$, $p = 0.032$) (Figure 7A). This difference was most pronounced between D1 and D3 (Figure 7A) when re-epithelialization was complete in paedomorphs but not in metamorphs.

In order to further quantify the inflammatory response we examined neutrophil infiltration in paedomorphs and metamorphs. Using a polyclonal antibody to myloperoxidase (MPO) we counted all neutrophils in 5μm sections of the wound bed between the epidermis and underlying muscle following injury (Figure 7B). Neutrophils were present in low numbers following injury (compared to mammals) and were not significantly different between paedomorphs (n = 8) and metamorphs (n = 8) ($F = 2.50$, $p = 0.136$). Examining MPO positive cells across all axolotls (n = 16) the effect of days post injury approached significance ($F = 3.04$, $p = 0.058$) and post-hoc comparisons

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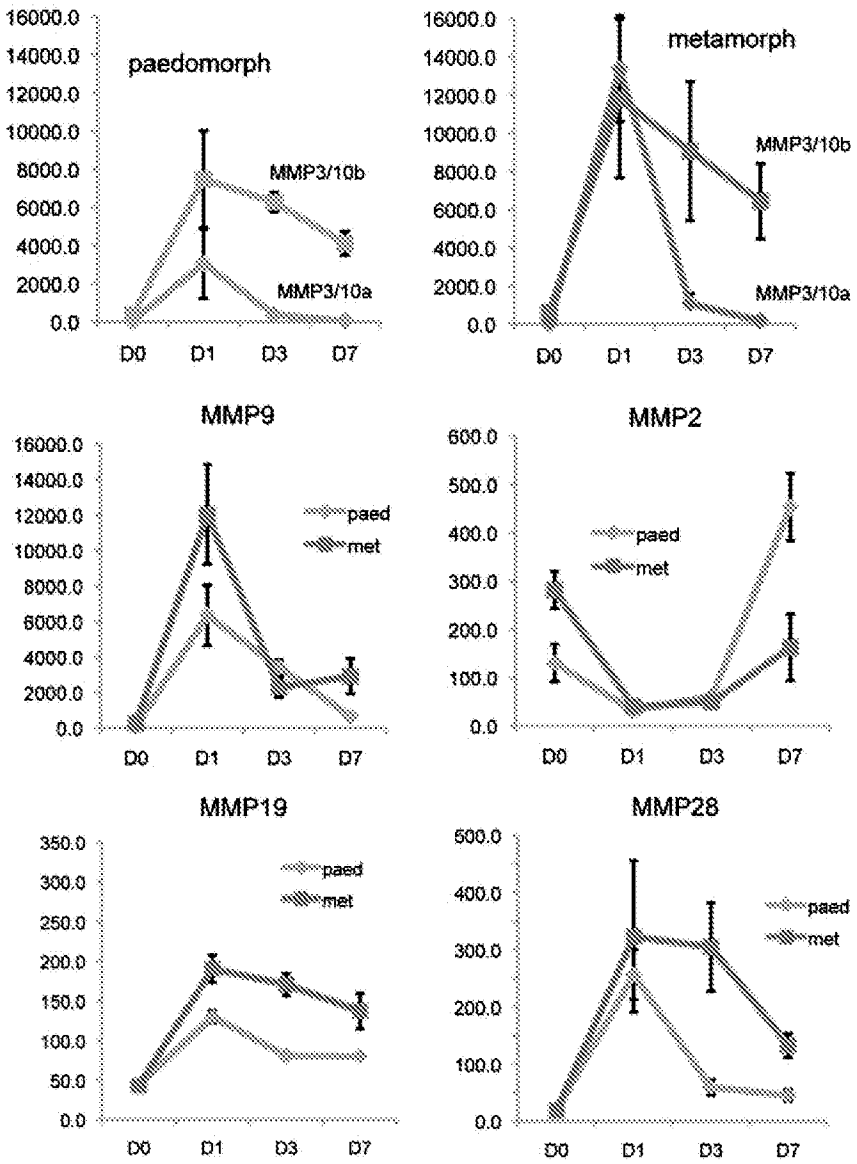


Figure 6. ***Matrix metalloproteinase*** **(*MMP*) expression during re-epithelialization and new tissue formation.** Expression values (y-axis) reflect absolute expression values from Affymetrix axolotl genechips (see Table S1 for exact values). Error bars represent standard error. Blue lines represent paedomorphs and red lines represent metamorphs. Expression kinetics for selected *MMPs* generally followed two patterns; (1) a strong upregulation at D1 followed by an equally strong decrease at D3 and a continued decrease or leveling off at D7 [*MMP3/10a, MMP9*] and (2) a strong upregulation at D1 which remained high at D3 and then downregulated at D7 [*MMP3/10b, MMP19*]. For *MMP28* the kinetics followed pattern 1 for paedomorphs and pattern 2 for metamorphs suggesting that *MMP28* expression was highly connected to re-epithelialization. The expression profile for *MMP2* was unique in that is was downregulated following injury and was upregulated after re-epithelialization was complete.
doi:10.1371/journal.pone.0032875.g006

revealed a significant decrease in neutrophil levels at D7 ($t = 2.014$, $p = 0.0226$).

In mammalian wounds, neutrophils represent the majority of leukocytes present during the early inflammatory response and comprise approximately 59% of circulating leukocytes [38]. Our data suggested that neutrophils comprised a relatively small fraction of infiltrating leukocytes in axolotls after injury, and we asked if this was due in part to low levels in circulation. Using a modified Wright's stain to identify leukocytes and Sudan Black to accurately identify the neutrophil population, we calculated leukocyte profiles (percentage of total leukocytes) for both paedomorphs ($n = 5$) and metamorphs ($n = 5$) (Figure 7C and Table S2). We found that leukocytes comprise approximately 4% of circulating blood cells with neutrophils accounting for ~21% of the leukocyte population in both forms (Table S2). Comparing paedomorphs and metamorphs we found significantly higher eosinophil levels in paedomorphs ($t = 2.49$, $p = 0.043$) and significantly higher lymphocyte levels in metamorphs ($t = 3.96$, $p = 0.004$). Taken together, these results show that axolotls display an influx of leukocytes following full thickness excisional wounding demonstrating an inflammatory response to wounding. However, relative to mammals, neutrophils comprise a smaller fraction of inflammatory cells at the wound site, and this correlates with their relatively lower representation in circulating blood.

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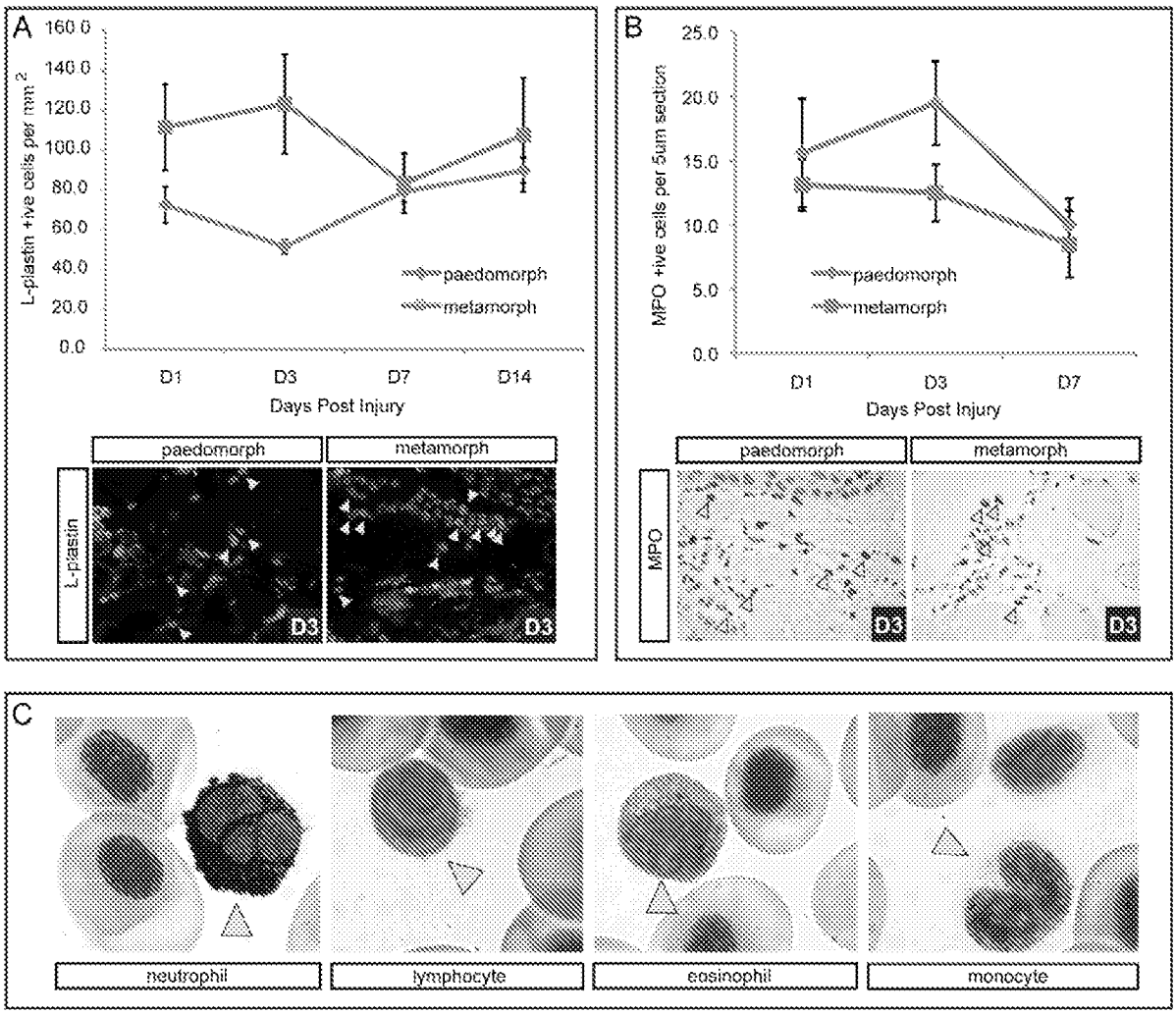


Figure 7. Higher initial leukocyte levels are present in terrestrial axolotls coincident with slower re-epithelialization, but neutrophil levels are not different between morphs. L-plastin was used as a pan-leukocytic marker to quantify total leukocyte levels following injury. A) Total leukocytes counted per mm^2 in the wound bed at 1, 3, 7 and 14 dpi (n = 4 for each morph). L-plastin positive cells are red (yellow arrows), nuclei are stained blue (Hoescht) and green fluorescence was used to account for autofluorescencing erythrocytes that were excluded as leukocytes. An influx of leukocytes was observed 24 hrs dpi, with higher numbers present in terrestrial axolotls. Leukocyte levels dropped in metamorphs at D7 and converged with paedomorph levels. Levels for paedomorphs were not significantly changed after D1. B) All neutrophils present in the wound bed (yellow arrows) were counted on 5 μm sections above the muscle using myloperoxidase (n = 8 for each morph; see Figure S6 for positive control staining in liver). Neutrophil levels were generally low and were not significantly different between paedomorphs and metamorphs, although they did drop significantly at D7. C) Modified Wright-Giemsa stain used to indentify individual leukocytes in circulating axolotl blood. Sudan black was used to stain neutrophils. Yellow arrows indicate the specific leukocyte type.
doi:10.1371/journal.pone.0032875.g007

Percent Wound Closure Mimics Contraction Rates During Healing of Human Skin Wounds

Wound contraction is an important component of the repair process and is an important metric when comparing wound repair across taxa [39]. In humans and pigs, contraction of circular wounds accounts for less than 50% of wound closure, while in rodents contraction accounts for ~90% of repair [39]. Following excisional wounding, the wound area increased slightly in metamorphs while remaining constant in paedomorphs and after 24hrs the wounds in both groups contracted at approximately the same rate (Figure 8A). Quantifying the rate of wound contraction between paedomorphs and metamorphs, we found that percent contraction was significantly greater in paedomorphs and resulted in a greater degree of wound closure (paedomorphs = 67% ±4.4% and metamorphs = 37.9% ±9%) (Figure 8B). In fact, our analysis shows that metamorphic axolotls contract their wounds to approximately the same degree as human skin after wounding.

Prior to wound contraction, some dermal fibroblasts in the granulation tissue and at the wound margins begin to express smooth muscle actin and are responsible for generating contractile forces that pull the wound margins together [40,41]. We also examined localization of alpha-smooth muscle actin (alpha-SMA) (a marker of myofibroblasts) in paedomorphs and compared its distribution to mice at similar time points after wounding (Fig. 8C). Approximately 10 days after injury, mice showed a high number of alpha-SMA positive cells at the wound margins when the wound area begins to rapidly contract (Figure 8C; white circled area). In contrast, we did not detect many alpha-SMA positive cells in axolotls at the wound margins or in the wound bed at D10, although the basement membrane beneath the wound epidermis

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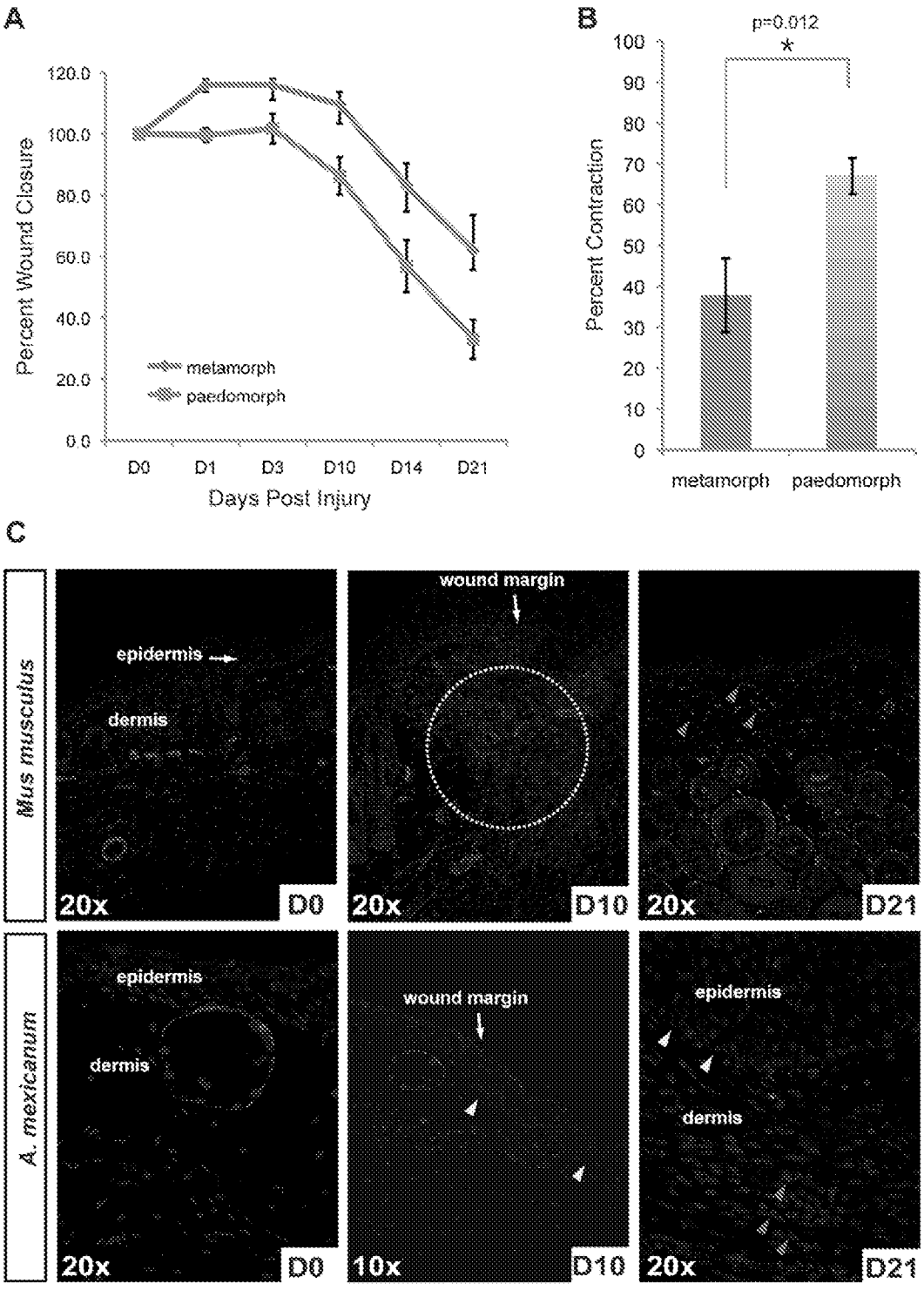


Figure 8. Contraction during scar-free healing in axolotls is similar to tight-skinned mammals. A) Percent wound closure in paedomorphs and metamorphs over 21 days (when contraction is complete). Metamorph wounds expanded by 10% following wounding and wounds contracted at about the same rate in both morphs. B) Paedomorph wounds contracted ~27% more than metamorph wounds with contraction accounting for 37.9% of wound closure in metamorphs. C) Alpha-smooth muscle actin (alpha-SMA) localization in unwounded skin and during wound repair in *Mus musculus* and axolotls (*A. mexicanum*) to identify myofibroblasts. Alpha-SMA localized to blood vessels and a few cells in mouse skin and around glands and the stratum compactum in axolotl skin. Ten days post injury (dpi), when contraction rates are highest in mouse wounds, alpha-SMA positive cells are detected at high levels at the wound margins (white dotted circle). In contrast, we detected very few alpha-SMA positive cells ten days after wounding in axolotl tissue. We did, however, detect alpha-SMA in the regenerating basement membrane (yellow arrows). Twenty-one dpi we detected a high number of myofibroblasts in mouse tissue (red arrows). We detected a few myofibroblasts in axolotl tissue at D21 (red arrows) near the underlying muscle.
doi:10.1371/journal.pone.0032875.g008

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appeared positive for alpha-SMA (Figure 8C). We observed numerous alpha-SMA positive cells at D21 in mouse granulation tissue compared with relatively few in axolotl provisional matrix. Taken together, these data show that wound contraction in axolotls mimic wound contraction in human skin, although there are relatively few myofibroblasts present during axolotl skin regeneration.

Dermal regeneration is Characterized by High Levels of Tenascin-C

The process of dermal repair in mammals (which ultimately results in a scar) proceeds via production of a fibrin clot, (referred to as the provisional extracellular matrix), degradation and replacement of provisional matrix by granulation tissue, and remodeling of granulation tissue into a fibrotic scar [1]. Following re-epithelialization in paedomorphs (D1) and metamorphs (D3), the wound bed was rich in blood cells and plasma but no scab formed (Figure 2). Provisional matrix in mammals is rich in fibronectin and thrombospondin [1] and along with plasma we detected low levels of fibronectin at the wound margins and beneath the wound epidermis at D7 in both morphs (Figure 9A). In contrast, we were unable to detect appreciable collagen deposition at D7 using histochemical techniques (Figure 1 and Figure 3). During mammalian wound healing, deposition of granulation tissue matrix proceeds in the stereotypical sequence of fibronectin, collagen type III and collagen type I [42]. At D14 fibronectin was present in low concentrations in granulation tissue in both morphs (Figure 9A). Using picrosirius red to detect both collagen type III and collagen type I, we predominantly observed collagen type III beneath the epidermis at D14 (Figure 10). As collagen synthesis continued collagen type III was replaced with collagen type I and as new dermis became progressively acellular, the density and diameter of collagen type I fibers increased (Figure 10). Fibronectin remained detectable in granulation tissue (D21), albeit at very low levels (Figure 9A).

While high levels of fibronectin and collagen are present in granulation tissue and also fibrotic scar tissue, the appearance of tenascin-C (TN-C) during mammalian wound healing is normally restricted to the wound margins and thought to stimulate cell migration. Concomitant with these observations we found intense TN-C protein localization at the wound margins and beneath the epidermis at D7 (Figure 9B). However, as dermal regeneration proceeded, TN-C persisted in high amounts throughout the wound bed and within regenerating muscle (Figure 9B). This association was particularly striking in the underlying muscle where TN-C formed a sharp boundary between regenerating and undamaged muscle (Figure 9B). TN-C levels remained high until the dermis had regenerated (Figure 9B). Taken together these findings suggest that collagen synthesis during axolotl scar-free healing proceeds similarly to that which is observed during mammalian wound repair and low levels of fibronectin and persistent high levels of TN-C characterize the anti-scarring matrix.

Discussion

Although many salamanders are capable of complete appendage regeneration (e.g. limb, tail), a recurring question is whether wounds made on the body heal with a scar or instead, heal scar-free [32]. In this study we demonstrate complete and perfect regeneration of adult axolotl skin following full thickness excisional (FTE) wounding to the dorsal flank. Additionally, we tested the hypothesis that loss of larval skin characters in metamorphic axolotls results in scarring following FTE wounding. Instead, we find that metamorphic axolotls are capable of scar-free healing. To our knowledge, this is the first demonstration of perfect scar-free healing of non-limb FTE wounds in an aquatic or terrestrial adult vertebrate. Comparing skin regeneration in paedomorphic (aquatic) and metamorphic (terrestrial) axolotls, metamorphic axolotls exhibited slower re-epithelialization, increased numbers of leukocytes during the early inflammatory response, increased deposition of extracellular matrix (ECM) and an almost doubling in the time required for complete skin regeneration. Compared to mammalian wound repair, terrestrial axolotls exhibited a reduced hemostatic response, lower neutrophils levels, similar duration of inflammation, faster time to complete re-epithelialization, a delay in new transitional matrix production and differences in the relative composition of the new ECM (Figure 11). These data suggest that further exploration of FTE wounding in axolotls is an excellent model to investigate the cellular and molecular regulation of scar-free healing.

Hemostasis

Wound healing in mammals proceeds along a stereotypical timeline consisting of repair processes that overlap in time and space and these processes have generally been included within three general phases; inflammation, new tissue formation and tissue remodeling [1,6,43]. Wound healing begins immediately after injury through the extravasation of blood products and activation of the coagulation cascade. In mammals, subsequent hemostasis is achieved through formation of a thick clot formed from platelets and plasma derived fibrin. This clot acts as a temporary plug and as a reservoir of growth factors, while the fibrin matrix acts as a scaffold for infiltrating inflammatory cells. In aquatic axolotls, the hemostatic response appeared limited with a thin layer of coagulated plasma at the wound site that was mostly devoid of cells. Following re-epithelialization, the neoepidermis was in close proximity to the underlying muscle with little evidence of a persistent fibrin clot prior to new ECM production. The presence of a clot in terrestrial axolotls was more pronounced, but was still greatly reduced compared to mammals. Erythrocytes and plasma formed the majority of material above the fragmenting muscle and it was unclear to what extent fibrin contributed to the loosely aggregated clot matrix. Following re-epithelialization, some blood plasma, erythrocytes and leukocytes were observed above and within the fragmenting muscle fibers, but the extent of a clot remained minimal and the epidermis remained in close proximity to the underlying muscle. Taken together, these findings show that axolotls restore hemostasis quickly and without formation of an extensive fibrin clot.

During mammalian wound healing, platelets degranulate, initiate the coagulation cascade and provide a wealth of growth factors and chemokines (e.g. PDGF, TGFß, VEGF, EGF, and IGF) that activate mesenchymal cells and attract inflammatory cells to the wound site [7,44]. Although some evidence suggests that platelets are not required during wound healing in mammals [45], all of these molecules have been shown to play a role during wound repair [46]. The degranulating action of amphibian platelets (thrombocytes) during injury is poorly understood, as are the growth factors produced during hemostasis. Future studies investigating the relative complement of chemoattractants and mitogens present in the clot matrix during scar-free healing in axolotls will shed light on how this molecular cocktail might direct the early events of wound healing towards regeneration in lieu of scarring.

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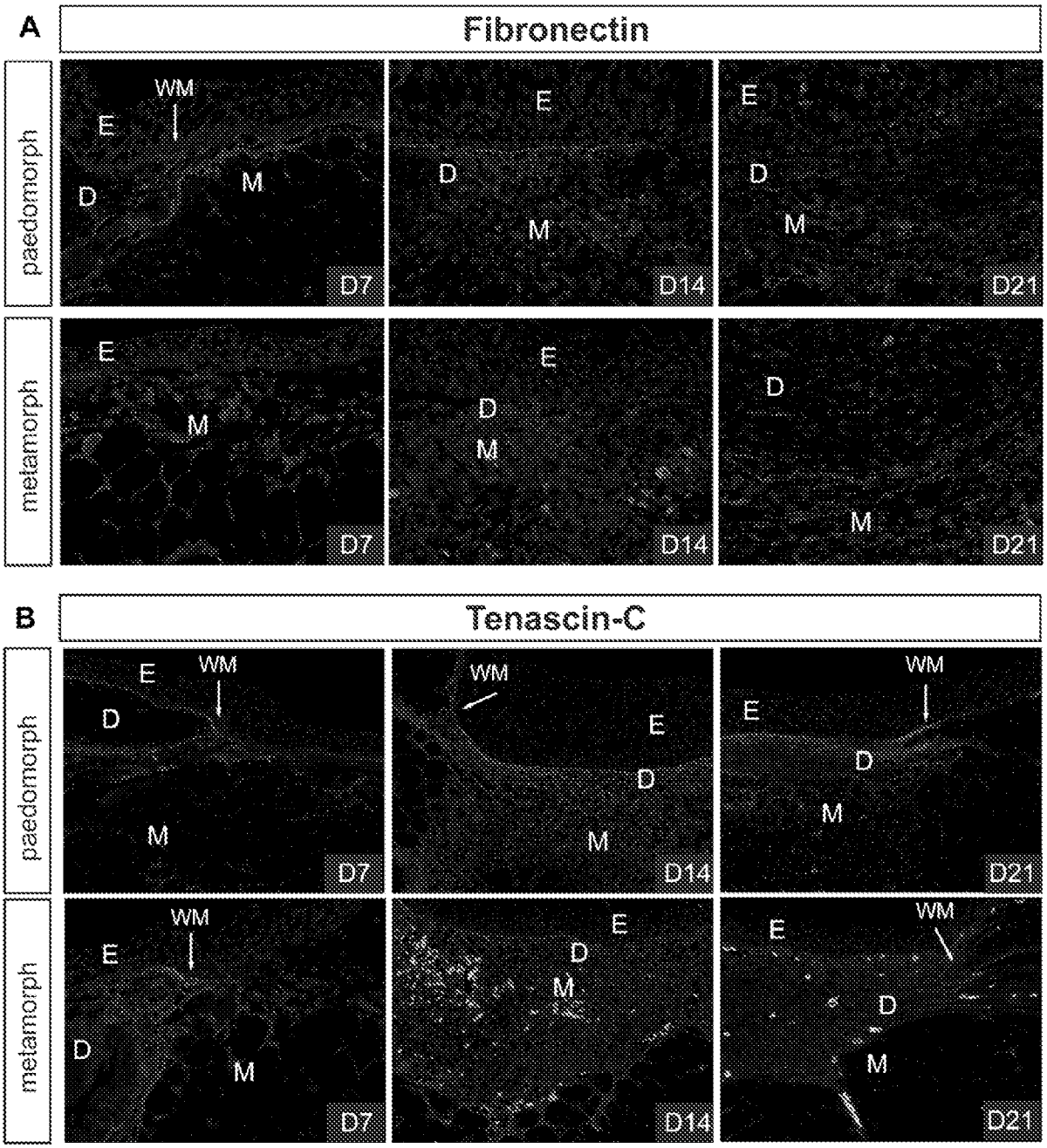


Figure 9. Regenerative ECM in axolotl wounds is characterized by high levels of tenascin-C. A-B) Fibronectin (FN) and tenascin-C (TN-C) levels were detected during scar-free healing in paedomorphs and metamorphs using an antibody to axolotl fibronectin and a polyclonal antibody to chick tenascin-C. We detected low levels of FN in the basement membrane at D7, and at the wound margins in both morphs. FN was present during ECM deposition at D14 in the center of the wound bed, but in relatively small amounts. By D21 little FN persisted in the regenerating dermis. B) TN-C was detected at the wound margins, in the basement membrane and surrounding some cells at D7. Fourteen days post injury we detected high levels of TN-C throughout the wound bed and in regenerating muscle. A sharp boundary formed between intact muscle and regenerating muscle. These high levels of TN-C persisted during dermis regeneration. Green fluorescence was used to detect autofluorescing erythrocytes. Epidermis (E), dermis (D), muscle (M), wound margin (WM).
doi:10.1371/journal.pone.0032875.g009

Inflammation

Including hemostasis, the sequential influx and action of neutrophils, macrophages and lymphocytes constitutes the inflammatory phase of wound repair. In mammalian wounds, neutrophils are the predominant cell type present immediately after injury and are necessary to destroy bacteria and combat infection. The recruitment of neutrophils occurs passively (through plasma aggregation) and via chemotaxis in response to the activation of complement, degranulating platelets, and from bacterial degradation. Neutrophils in turn have the capacity to attract additional inflammatory cells and amplify the inflammatory response. Monocytes arrive at the wound site 2–3 days after injury and transform into macrophages. Macrophages serve to remove neutrophils and cellular debris, which helps to resolve inflamma-

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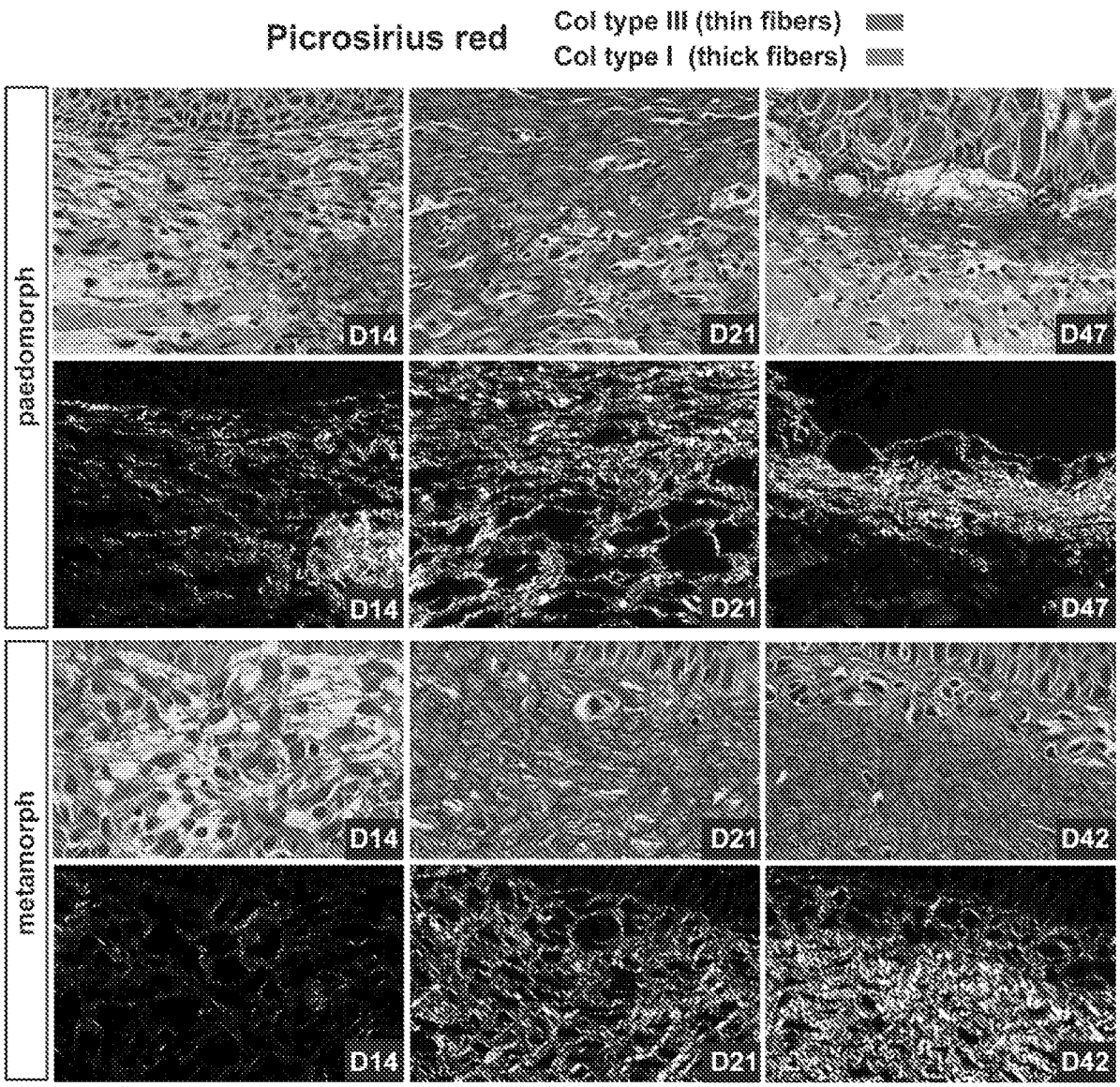


Figure 10. Collagen type III predominates early during new tissue formation and is slowly replaced by collagen type I during scar-free healing. Picrosirius red staining was used to detect collagen type III and collagen type I during scar-free healing in the wound bed. Using polarized light to detect bifringence, collagen type III (green fibers) was deposited first during new ECM deposition in both morphs. As the dermis regenerated, collagen type III was slowly replaced by collagen type I (red fibers) in both morphs.
doi:10.1371/journal.pone.0032875.g010

tion, and also are thought to assist later during new tissue formation to regulate the balance between fibrosis and scarring [36]. Following FTE wounding in aquatic and terrestrial axolotls we detected neutrophils 24 hrs after injury. Neutrophil levels, however, were reduced compared to equal size FTE wounds in mammals suggesting a reduction through passive aggregation or chemotaxis [47]. Compared to mammals, which maintain ~60% of their circulating leukocytes as neutrophils, we found axolotls maintain approximately ~21% of their circulating leukocytes as neutrophils and this suggests that low neutrophil numbers at the wound site result partly from low numbers in circulation. In support of this finding, a recent study in larval axolotls found very few neutrophils in partial thickness tail wounds (which incur very little bleeding), while stab wounds made into the tail muscle, led to greater numbers at the wound site [26]. Neutrophil depletion studies in mammals have shown that as long as conditions remain sterile, neutrophils are not required during wound healing, and their loss may actually increase the rate of re-epithelialization [48,49]. Our findings show that their presence, albeit in low numbers, is compatible with scar-free healing and is coincident with a faster rate of re-epithelialization compared to mammalian wounds.

Although we did not examine additional individual leukocytic lineages, comparing L-plastin positive cells (a pan-leukocytic marker) in aquatic and terrestrial axolotls, we found higher total leukocyte numbers persisting in terrestrial animals while the wound bed remained exposed. Following re-epithelialization, total leukocyte numbers converged for both morphs suggesting that the early influx of inflammatory cells may not significantly influence the subsequent arrival of inflammatory cells. Together, our finding that total leukocyte levels remain high at least 14 days post injury

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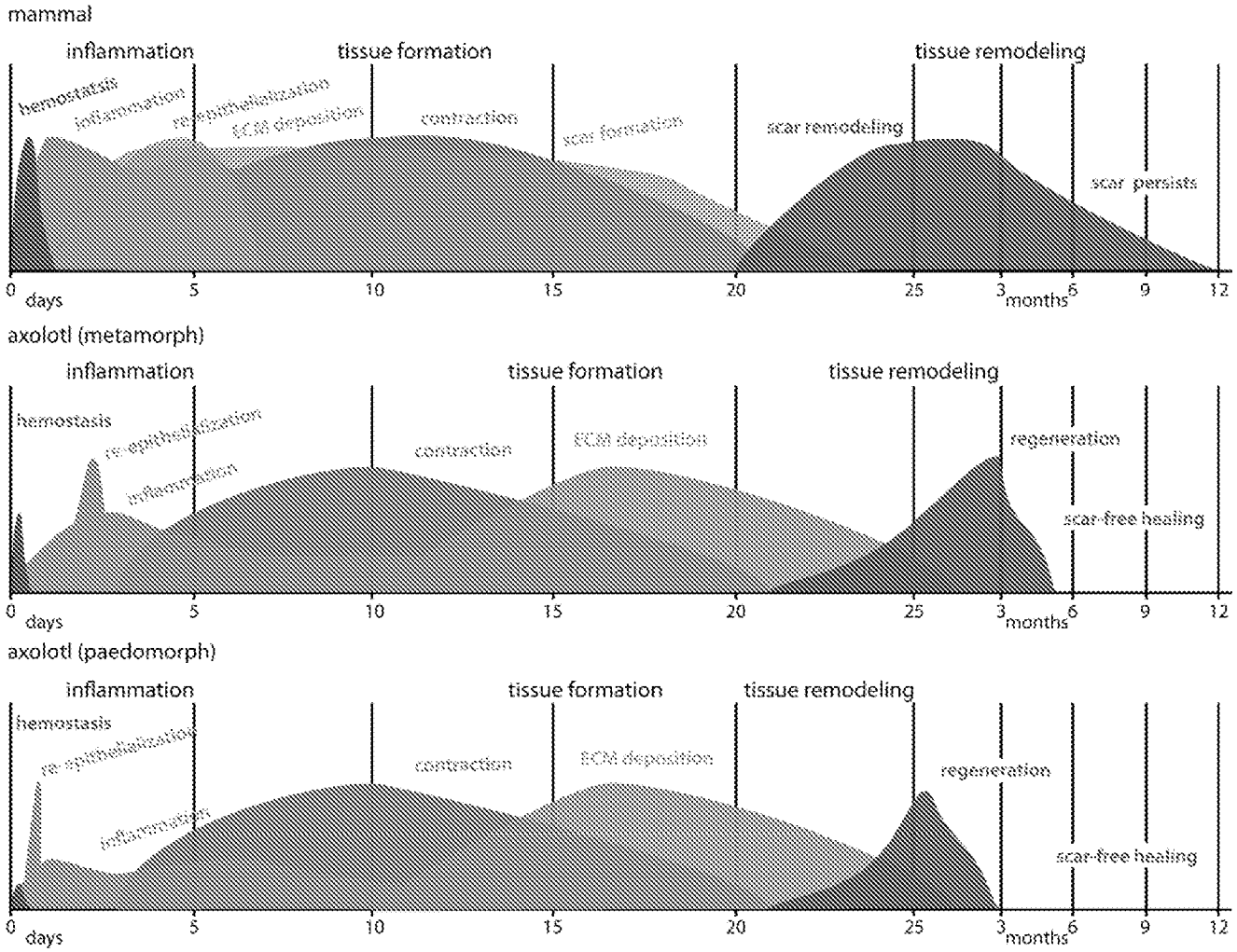


Figure 11. Summary of wound healing processes comparing axolotls and mammals. The x-axis represents time and the y-axis represents percent maximal response for each process. Information for mammals has been approximated from the literature and from our own experiments with 4mm FTE wounds in mice. Colors represent individual processes overlapping across the three phases of wound healing; inflammation, new tissue formation and tissue remodeling. Comparing paedomorphic and metamorphic axolotls, metamorphs exhibited an increased hemostatic response, slower re-epithelialization, increased early inflammatory response, increased and prolonged deposition of extracellular matrix (ECM) and an almost doubling in the time required for complete skin regeneration. Comparing scar-free healing in terrestrial axolotls to scar formation in mammals, terrestrial axolotls exhibited a reduced hemostatic response, lower neutrophil levels, faster re-epithelialization rate, delay in ECM production, differences in the relative composition of the new ECM, regeneration of glands and dermis regeneration instead of scarring. Schematic for mammals is adapted from Mikael Häggström.
doi:10.1371/journal.pone.0032875.g011

supports the role of an active inflammatory phase during scar-free healing in axolotls.

The notion that reduced inflammation promotes regeneration in lieu of scarring has been popular, but evidence from mammals and amphibians remains inconclusive [50,51]. Aspects of limb regeneration in adult anurans, which lose the ability for limb regeneration following metamorphosis, can be stimulated through tissue aggravation [52] and chemical irritation [53], suggesting that prolonging tissue inflammation can prime a dormant regenerative response. On the other hand, scar-free healing in fetal mammals is correlated with reduced inflammation prior to their development of a mature immune system (compared to scarring in adults) [10,54]. Data from adult mammals suggests platelets [45], neutrophils [48] and macrophages [55] are dispensable for wound repair, but their removal does not lead to scar-free healing suggesting that reducing inflammation alone will not induce a regenerative response. The existing data from chronic and hypertrophic wounds support the hypothesis that too much inflammation promotes excessive fibrosis, and in the context of our results, it is likely that inflammation must not exceed a threshold level for scar-free healing to occur [7,56]. A recent study designed to increase inflammation in axolotls by injecting bleomycin following partial thickness tail wounds, found evidence of increased fibrosis following thirty days of administering the drug during the healing process [26]. While it is tempting to speculate that this exposure might lead to scarring, given the continuous exposure to bleomycin prior to tissue harvest it is difficult to interpret their results as they relate to heightened inflammation early during the healing process. Future experiments over-stimulating inflammation in our terrestrial FTE model of scar-free healing through chemical and molecular methods during the naturally occurring window of inflammation will allow us to

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examine the cellular and molecular mechanisms that connect inflammation and fibrosis. Our findings reinforce the idea that inflammation is not altogether anti-regenerative and support a role for an active inflammatory response during scar-free healing.

Re-epithelialization

As the inflammatory phase begins to wind down, keratinocytes at the wound-edge migrate to re-epithelialize the wound bed and restore the physical barrier separating the external environment. Concomitantly, granulation tissue (transitional matrix) is produced to replace the fibrin clot and together these processes will contribute to new tissue formation at the wound site. We found that adult paedomorphic axolotls completely re-epithelialized 4mm wounds 18–24 hrs post injury, while terrestrial axolotls exhibited slower re-epithelialization. In terrestrial axolotl skin, wound edge keratinocytes migrated after a delay period of ~24 hrs and complete re-epithelialization occurred up to 3 days after injury. This finding suggests that the transition from a pseudo-stratified to stratified epidermis following metamorphosis prevents rapid re-epithelialization in response to injury. Previous research in newt limbs suggested that epidermal keratinocytes are constitutively active and thus are primed to migrate following injury [24]. Our finding in paedomorphs supports this conclusion, while the observed delay in terrestrial axolotls demonstrates the need for keratinocyte activation as occurs in mammalian wound healing [57]. Interestingly, the onset of keratinocyte migration in mammals occurs after a delay period of 18–24 hrs [57,58], which is identical to what we observe in terrestrial axolotls. FTE wounds made in fetal rats that heal scar-free (E16), or result in scarring (E19), both re-epithelialize in 72hrs and together with our data in terrestrial axolotls, suggest that the speed of re-epithelialization cannot alone explain the ability to perfectly regenerate skin [13]. Our results suggest that rapid re-epithelialization reduces the time required to regenerate skin, and may contribute to reduced inflammation. Once activated, terrestrial axolotl keratinocytes appeared to migrate rapidly across the wound bed and it would be interesting to compare the rate of re-epithelial migration to similar sized mammalian wounds where moisture prevents scab formation. Comparing epidermal cells between these two morphs during wound healing will provide a powerful model to identify the molecular control of keratinocyte activation and will be useful in identifying new molecules that might stimulate keratinocyte migration in non-healing chronic wounds.

ECM Production and New Tissue Formation

While a dampened hemostatic response, reduced inflammation and quicker rate of re-epithelialization likely contributes to scar-free healing in axolotls (compared to scarring in adult mammals), the slow deposition of new extracellular matrix (ECM) and its unique molecular composition suggests newly synthesized axolotl ECM may antagonize fibrosis and promote regeneration (Figure 11). Shortly after re-epithelialization begins during mammalian wound repair, the provisional matrix, which is rich in cross-linked plasma fibronectin, is replaced by granulation tissue rich in macrophages, fibroblasts, newly synthesized ECM molecules and neovasculature [59]. Within this granulation tissue, fibroblasts secrete fibronectin, collagen type III and collagen type I, and this matrix will form the transitional ECM that will ultimately be remodeled into dermal scar tissue [1,60]. Although fibronectin and collagen are the most abundant ECM molecules in mammalian granulation tissue, tenascin-C is also deposited, first at the wound margins, then at the epidermal-dermal junction of migrating keratinocytes, and finally, within the granulation tissue itself [61]. During scar-free healing in terrestrial axolotls the wound bed at the epidermal-dermal junction contained little new ECM for 10 days after re-epithelialization. This delay period was striking in its duration and our finding that the neoepidermis was a potent source of several matrix metalloproteinases (MMPs) suggests an active inhibition of new ECM production. While we found strong and rapid upregulation of several *MMPs* (*MMP1, axCol, MMP3/10a,b, MMP19, MMP28, MMP9*) prior to, and during re-epithelialization, most of these returned near baseline levels following re-epithelialization supporting their role during keratinocyte migration [25]. However, *MMP3/10b* and *MMP9* persisted at relatively high levels in the neoepidermis, and *MMP2* levels began increasing after re-epithelialization. All three of the homologous mammalian proteinases can degrade fibronectin, and MMP3 and MMP2 are capable of degrading collagen type III and type I [62,63]. Together these data suggest that during scar-free healing in axolotls, persistently high MMP levels may act after re-epithelialization to prevent new ECM production as resident tissue undergoes histolysis and the wound bed is prepared for deposition of the regenerative matrix. Supporting this notion, broad inhibition of MMPs during newt limb regeneration can lead to acellular scar tissue formation and studies during scar-free healing in fetal wounds have observed higher MMP levels compared to adult wounds [13,34]. Ongoing studies in our laboratory are targeting these individual MMPs and their specific activities towards understanding how they regulate both re-epithelialization and the fibrotic response during scar-free healing.

The role of other proteinases such as the ADAMs and ADAMTSs during mammalian wound healing is poorly understood, while their role during fetal wound healing is completely unknown. Their ability to cleave membrane-bound domains of a host of different proteins (e.g. cytokines, growth factors, cytokine and growth factor receptors) thus releasing them into the extracellular space, has driven speculation that these factors may play an important role during wound healing, although direct studies are lacking [64]. Only ADAM9 and ADAMTS1 have been found to have a direct role during wound healing, with faster re-epithelialization occurring in mouse knockout studies of both individual genes [65,66]. During scar-free healing in axolotls we found a small but significant increase in *ADAM9* following injury but did not detect changes above baseline in any of the other *ADAM* or *ADAMTS* genes. Together, our findings suggest that ADAMs may not play a vital role during scar-free healing, although they are likely to work synergistically during keratinocyte migration.

Comparing our ECM data with that acquired from studies on scar-free healing in fetal mammals, both similarities and differences can be noted. Although there has been considerable controversy surrounding the production of collagens in fetal wounds, owing to the variety of species, wound models and fetal age, it is generally accepted that collagens are produced during fetal wound healing and are produced more rapidly compared to adult wounds [67,68]. While collagen fibrils are deposited in dense bundles parallel to the wound bed, fetal collagen fibrils appear to be deposited in a reticular fashion, almost identical to the surrounding dermis [68]. This contrasts with the situation during scar-free healing in axolotls where collagen deposition was delayed (as in adult mammals) and also occurred in dense bundles throughout the wound bed. Thus our data suggests that the pattern of collagen deposition observed during adult scarring is not technically incongruent with regeneration and may suggest the lack of later remodeling events or differences in the structural composition of the collagen deposited.

The appearance of new ECM during axolotl wound healing was coincident with fibroblast infiltration beginning at D14, and small

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amounts of fibronectin production. Similarly, fibronectin is recognized as a prominent component of the provisional matrix during adult and fetal wound healing, appearing early associated with the fibrin clot and then slowly disappearing concomitant with the deposition of collagen [60,68]. It is expressed earlier in fetal wounds compared to adult wounds and its disappearance is correlated with the onset of collagen deposition. It is also present at high levels in unwounded fetal skin compared to adults [69]. In contrast to mammalian wounds, axolotl transitional matrix was highly enriched with tenascin-C. Fibronectin appeared transiently with collagen type III in the wound bed while tenascin-C persisted through D42 as collagen type I was produced and the dermis regenerated. The role of tenascin-C during fetal wound healing has been poorly studied, although in the same study that examined fibronectin distribution during incisional healing in the lip [68] tenascin-C levels were found to be slightly higher in the fetal versus the adult. Some data suggests that tenascin-C can antagonize certain functions of fibronectin including T-cell activation [70] and in the context of fetal scar-free healing and axolotl scar-free healing suggests that the relative concentration of each in the extracellular matrix might act to regulate an adaptive immune response. Tenascin-C is thought to behave as an anti-adhesive molecule promoting proliferation and migration over differentiation and as such, is a transient molecule during mammalian wound healing [1]. Tenascin-C can disrupt cell-matrix interactions and has been speculated to maintain monocytes and blastemal cells in a de-differentiated state during salamander limb regeneration [23,71,72]. These data suggest that the composition of ECM produced during a regenerative response may have an anti-fibrotic effect that helps to recapitulate embryonic development and promote a regenerative response.

Remodeling and Regeneration

The evolutionary process that has led to wound repair while efficient, is not functionally perfect. Epidermal appendages do not regenerate and the uninjured dermal architecture is replaced by dense parallel bundles of collagen that reduce the mechanical properties of normal skin. While the wound bed ECM during mammalian wound repair will eventually result in a fibrotic scar, we observed complete regeneration of not only dermal layers but of epithelial derived glands. The regeneration of hair follicles has been observed in rabbit wounds and ear punches and in very large wounds made on young mice [73,74,75]. Other than these reports, the regeneration of epithelial-derived structures in adult vertebrates has not been observed. Although hair follicle and gland development is well understood in mammals, it is poorly understood at the molecular level in amphibians. Ongoing experiments in our laboratory examining the molecular control of gland development are underway and it will be interesting to determine how regeneration of these structures is controlled following scar-free healing [76]. Specifically, it will be of interest to determine the location of inductive signals that lead to the specification of new glands and whether these signals are localized or ubiquitous during the regenerative process.

Axolotl Model of Scar-free Healing

The data in this paper establishes a novel, adult model of perfect skin regeneration in which to test hypotheses about the molecular regulation of fibrosis and more generally, the underlying genetic control of scar-free healing. As such, we have developed an FTE wound model in both aquatic and terrestrial axolotls and characterized their ability to perfectly regenerate all damaged tissues. To date no *adult* animal model has been described that is capable of perfectly healing FTE wounds and underlying muscle (outside of the limb and tail). While numerous studies have described healing without scarring in fetal mammals and marsupials [10], the fetal model provides a number of drawbacks that the axolotl model overcomes. First, the fetus is still developing in the womb and this creates both biological and practical complications. The developing fetus, at the time when it heals scar-free, has an immature endocrine system, is immunoincompetent, is contained in a moist sterile environment, and its cells are in a state of chronic hypoxia [16]. In contrast to fetal models, terrestrial axolotls are fully developed, sexually mature, have a fully developed immune system (albeit not as sophisticated as adult mammals), and their FTE wounds are desiccating and open to infection. From a practical standpoint, "in utero" surgery is not required, up to six 4 mm FTE wounds can be made on the dorsal skin and pharmacologic drugs and chemicals can be easily delivered to antagonize or agonize signaling pathways. In combination with their ability to perfectly regenerate all tissues damaged in these wounds (e.g. epidermis, dermis, glands, nerves, muscle) this model shifts experimentation into a system where the ultimate clinical outcome actually occurs.

This new animal model will provide investigators a new paradigm in which to address the multiple processes of wound repair (e.g. hemostasis, inflammation, keratinocyte activation and migration, ECM formation, tissue remodeling) in a truly scar-free healing adult, while also providing a tractable genetic system to investigate the cellular and molecular mechanisms that regulate these processes. Given the considerable body of literature on the molecular pathways and cellular processes involved during normal wound repair, understanding how these conserved molecular players operate in a pro-regenerative environment will lead to development of new therapies that target downstream effectors for testing in established mammalian models.

Materials and Methods

Animals and Wound Model

Ethics Statement. All procedures were conducted in accordance with, and approved by, the University of Florida Institutional Animal Care and Use Committee (IACUC Study #201101534 and #200903505).

Animals. *Ambystoma mexicanum* (Mexican axolotl) were acquired from the Ambystoma Genetic Stock Center (AGSC, Lexington KY) and bred in captivity. Animals were housed individually in Aquatic Habitats® (Aquatic Habitats Inc., Apopka, FL) flow-through systems at 21–23°C in Holtfreter's Solution and maintained on California blackworms (*Lumbriculus sp.*, J.F. Enterprises, Oakdale, CA). Animals were deemed adults after reaching sexual maturity (>9 months) and measured between 12–15 cm total length. Metamorphic (terrestrial) axolotls were acquired either directly from AGSC or transformed at the University of Florida through treatment with thyroxine [77]. In both cases metamorphosis occurred after animals had reached sexual maturity. Terrestrial axolotls were housed individually in 10-liter containers containing moist paper towels and fed nightcrawlers. Mice used in this study were Swiss Webster (Charles River) approximately 6-months old.

Full thickness excisional (FTE) wounding. Adult axolotls were anesthetized by full submersion in Benzocaine (Sigma) 0.01% (aquatic) or 0.02% (terrestrial). Sterile 4mm biopsy punches were used to create FTE wounds through the skin into the dorsal back muscle. In this way, 6 wounds were created on the dorsal surface posterior to the forelimbs and anterior to the hindlimbs. In order to harvest wound tissue at specific time-points, animals were anesthetized as above and the entire wound was harvested using a

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6mm biopsy punch and iridectomy scissors. FTE wounds in mice (through the panniculus carnosus) were made with 4mm biopsy punches through the dorsal skin posterior to the forelimbs and anterior to the hindlimbs.

Histology and Immunohistochemistry

For histological and immunohistochemical analysis, harvested tissues were fixed in 10% NBF at 4°C for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4°C and processed for paraffin embedding. Samples were cut at 5μm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4°C, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT. Frozen sections where cut at 12μm. For histological analysis, paraffin sections were stained with one of the following: Masson's Trichrome (Richard Allen Scientific) or Picrosirius Red (American Mastertech). For blood smears, blood was collected from limb amputations and air-dried on slides. Slides were stained with Sudan Black [78] to detect neutrophils and counterstained with a modified Wright-Giemsa Stain (Kodak).

Immunohistochemistry. Slides were rehydrated, antigen retrieval performed if necessary (see below for each antibody), washed in TBS, blocked for streptavidin and biotin (Vector Labs), and incubated with 1° antibody (see below) overnight at 4°C. The following day, slides were washed in TBS, incubated with biotinylated 2° antibody (Vector Labs) and subsequently incubated with streptavidin conjugated horseradish peroxidase antibody (Vector Labs) and visualized using DAB (3,3–diaminobenzidine) (Vector Labs) or incubated with a streptavidin conjugated Alexa-Flour 594 (Invitrogen). Images were captured on a Nikon Eclipse 6600 upright compound microscope using a Cool-Snap Pro true color camera (light microscopy) or an Olympus inverted microscope (IX81) with fluorescence using a Leica DFC310 FX (fluorescence microscopy).

Antibodies. For paraffin sections; Widespectrum Cytokeratin (DAKO; Z0622) 1:500, *Ambystoma* Fibronectin (gift of Thierry Darribere) 1:1000, Tenascin-C (Millipore, AB19013) 1:500, Collagen type IV (Rockland, 600-401-106-0.1) 1:450, alpha-smooth muscle actin (Abcam, ab5694) 1:100, Laminin (DAKO, Z0097) 1:500. For frozen sections; Myloperoxidase (Thermo, RB-373-A) 1:100, L-plastin (gift of Paul Martin) 1:2000. Antigen retrievals used were; microwave and Citrate Buffer (pH. 6.0) (Cytokeratin, Myloperoxidase, L-plastin, Tenascin-C, alpha-smooth muscle actin) or Proteinase-K treatment (DAKO) for 2 mins (Collagen type IV, Fibronectin, Laminin). Negative controls used isotyped IgG at the same concentration as the 1° antibody. A GFP filter was used to detect autofluorescence of erythrocytes.

Microarray Analysis

Epidermal tissue was harvested using a 4mm biopsy punch. Two wounds were made along the flank and posterior to the forelimbs. Harvested epidermis was pooled for each animal. Four biological replicates were collected from uninjured epidermis (D0) and at 1, 3, and 7 days post injury. RNA was isolated using Trizol Reagent (Invitrogen) followed by Qiagen RNeasy Clean-up columns (Qiagen). RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent). RNA processing for Affymetrix microarray analysis was performed at the University of Kentucky Microarray Core Facility and hybridized to 32, AMBY_002a520748F 2nd Generation Axolotl Affymetrix microarrays (Affymetrix) [79]. Microarray data was tested for quality control and analyzed in the software system R using the Bioconductor package and oneChannelGUI [80,81,82]. Microarray data was preprocessed, and normalized using RMA and a linear model testing the effect of time for each morph type was fit for each gene across all 32 GeneChips using limma [83,84]. Thus, two gene lists were produced containing significantly changed genes over time in paedomorphic or metamorphic animals. In these analyses, contrasts were made between each time point and a t-statistic was produced for each contrast. The moderated F-statistic calculated by the eBayes limma function [85] was used as an overall test for significance across time, similar to ANOVA. A gene was considered statistically significant if it had an FDR adjusted p-value <0.05 and changed at least 2 fold between any two time points. The microarray data is MIAME compliant and the CEL files are available on Sal-Site (www.ambystoma.org). In addition, the raw data is available through the public NCBI GEO database (accession number: GSE35255).

Statistics

For quantification of inflammation we used a 2-way ANOVA to examine the effects of morph (metamorph versus paedomorph) and day post injury on total leukocyte numbers (with individual as a random effect to allow for repeated sampling). For all other statistical analyses we used a Student's T-test.

Supporting Information

Figure S1 Axolotl (paedomorph) skin glands. A) Granular gland. B) Mucous gland.
(TIF)

Figure S2 Detailed aspects of dermis and gland regeneration. A) High magnification image of a gland regenerating and descending from the epidermis 44 dpi. B) Detail of wound margin (WM) showing the edge of the injured stratum compactum which is normally densely compacted and the loose collagen fibers beginning to coalesce in the wound bed. C) Detail of wound bed 180 dpi showing mature mucous gland and regenerated stratum compactum. Hypodermis (H) and dermis (D). Scale bars = 100 μm.
(TIF)

Figure S3 Detailed aspects of metamorphic axolotl skin regeneration over 147 days. A) Granular and mucous glands (yellow arrows) present in the stratum spongiosum. Collagen fibers are present between the glands. B) Some cellular aggregations in the epidermis appear to be early stages of regenerating glands (yellow arrows). C) Regenerated glands within densely compacted collagenous ECM beneath the epidermis. D) Stratum compactum is beginning to coalesce as the rest of the dermis has regenerated. Some fibrotic tissue remains within the regenerated muscle. E) Complete scar-free skin regeneration at D147 dpi. All tissue layers are present. Granular glands remain immature compared to uninjured skin. Scale bars = 100 μm.
(TIF)

Figure S4 Visualizing the rate of re-epithelialization in paedomorphic axolotls using GFP transplanted skin. 1.5 cm×1.5 cm squares of dermis and epidermis from ubiquitously expressing GFP axolotls were transplanted to same size explanted areas on the tail of adult white axolotls. After transplants had healed 4mm FTE biopsy punches were made through the transplanted tissue and GFP-labeled epidermis was observed and photographed migrating to cover the wound bed over 18hrs. Migration was observed beginning 3hrs post injury and was complete between 18 and 24hrs.
(TIF)

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Figure S5 *MMP* transcription and activity are correlated during tail regeneration in paedomorphs. A) Axolotl *MMP9* expression (semi-quantitative PCR) from tail tissue following amputation and through D14 post-amputation. Expression is downregulated 7 days post injury. B) Gelatin zymography was used to assess MMP activity over 21 days post injury. Red arrow points to tentatively assigned MMP9 position based on size (~85 kDa) from previously published work in newt and axolotl (Vinarasky et al. 2005, Santosh et al. 2011). C) Gelatinase activity lags behind transcriptional upregulation and peaks at D7. For quantification of activity, gel images were inverted, mean optical density of individual black bands were calculated on an Gel Logic gel imaging system (Kodak) and light was blocked to calculate a baseline (black) reference for each measurement. Standard errors are reported. Regenerating tail tissue and tissue 0.5mm rostral to the amputation plane was collected at 0hrs, 3hrs, 6hrs, 12hrs, 24hrs, 7 d, 14 d, and 21 d after injury (n = 3 per time point), snap frozen on dry ice, and stored at –80°C. Tissues were homogenized for 10 minutes on ice in 1:4 (w:v) homogenization solution [50 mol/L Tris-Cl (pH 7.6), 150 mol/L NaCl, 1% (v/v) Triton-X100, 10 mol/L EDTA, 1 mol/L PMSF], sonicated, set on ice for 10 minutes, and spun at 14 000×*g* for 10 minutes at 4°C. Supernatants were decanted off, quantified using a BCA protein assay kit (Pierce), and stored at –80°C. 25 µg total protein was diluted in zymogram sample buffer (Bio-Rad) and electrophoresed on Ready Gel zymogram polyacrylimide gels containing gelatin (Bio-Rad). MMP proteins were re-natured by washing gels in zymogram renaturation buffer (Bio-Rad) for 30 minutes and incubated for 16 hrs at 37°C in development buffer (Bio-Rad). Gels were stained with 0.5% (w/v) Coomassie Blue R-250 (Bio-Rad) for 30 minutes and de-stained with an acetic acid, methanol, and dH20 solution (1:5:4) until clear bands were visible.
(TIF)

Figure S6 Individual variation in leukocyte numbers during scar-free healing. A) Total leukocyte numbers based on L-plastin staining for paedomorphs and metamorphs (n = 4 for each morph). Because multiple wounds were made on the same animals the inflammatory response could be tracked per individual. B) One paedomorph exhibited an unusually high inflammatory response (paed 4) and we removed it from our analysis. C) Variation across metamorphs revealed no outliers. D) Control staining for myloperoxidase in axolotl liver section.
(TIF)

Table S1 Axolotl gene identifications based on first homologous human gene hit in BLAST. Expression values reflect absolute expression on genechip (max expression value = 30 k; expression values <100 = no expression). N = 4 animals per time point. D0P = day 0 paedomorph, etc. D0M = day 0 metamorph.
(XLS)

Table S2 Leukocyte profiles for *Ambystoma mexicanum* (paedomorph and metamorph). Numbers for each particular leukocyte type represented as a percentage of total leukocytes (thrombocytes were not counted).
(DOC)

Acknowledgments

We thank Sri Putta for help with bioinformatics, Marda Jorgenson for histological assistance and Ashley Stuart for animal care.

Author Contributions

Conceived and designed the experiments: AWS MM. Performed the experiments: AWS JRM MM. Analyzed the data: AWS JRM SRV MM. Wrote the paper: AWS. Commented and edited the manuscript: JRM SRV MM.

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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	1	of	2	GROUP ART UNIT: 1651	EXAMINER: Ruth A. Davis

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
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Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		WO	03/082201	A2	Anthrogenesis Corporation	10-09-2003	
		WO	2005/009498	A2	Scimed Life Systems, Inc.	02-03-2005	
		WO	2007/124127	A2	Wake Forest University Health Sciences	11-01-2007	
		WO	2012/131104		Ingell Technologies Holding B.V.	10-04-2012	
		WO	2012/131106		Ingell Technologies Holding B.V.	10-04-2012	

OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		Extended European Search Report for European Application No. 14737892.1 mailed September 28, 2016 (S1860.70000EP00).	
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FORM PTO-1449/A and B (modified PTO/SB/08) **INFORMATION DISCLOSURE STATEMENT BY APPLICANT**				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	2	of	2	GROUP ART UNIT: 1651	EXAMINER: Ruth A. Davis

		ZAIOU, Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. Journal of Molecular Medicine. 2007 Apr; 85(4):317-29. Epub 2007 Jan 10.	

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/RUTH A DAVIS/	01/10/2017

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4267880.1

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	1449	01/25/2017	S1860.70000US01
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UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Docket No.: S1860.70000US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	R. A. Davis
Art Unit:	1651

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AMENDMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This is responsive to the Office Action dated January 25, 2017. This response is filed with a Petition for a three-month extension of time (with fee).

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 10 of this paper.

AMENDMENTS TO THE CLAIMS

Please replace all prior listings of claims in this application with the following listing of claims.

1. (Withdrawn) A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. (Withdrawn) The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. (Withdrawn) The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. (Withdrawn) The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. (Currently amended) A tissue graft comprising extracellular matrix (ECM) components derived from a ~~urodele~~ Urodele, said graft derived by a process that comprises

(a) obtaining a tissue sample from a Urodele, which tissue sample comprises ECM connective tissue and cells; and

(b) decellularizing the sample to retain structural and functional integrity of the ECM connective tissue while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of ECM connective tissue as a xenograft.

6. (Withdrawn) An isolated, decellularized Urodele ECM.

7. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. (Withdrawn) The isolated ECM of claim 6, wherein the ECM includes basement membrane.

9. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. (Withdrawn) The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a sheet.

12. (Withdrawn) The isolated ECM of claim 11, wherein the sheet includes perforations.

13. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. (Withdrawn) The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

16. (Withdrawn) A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM

is a sheet of isolated, decellularized Urodele ECM.

18. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. (Currently amended) A sterile medical implant comprising sterile, isolated, ~~decellularized~~ Urodele Extracellular Matrix (ECM) ~~or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM~~ produced by a process comprising:

(a) obtaining a tissue sample from a Urodele, which tissue sample comprises ECM connective tissues and cells;

(b) decellularizing the sample to retain structural and functional integrity of the ECM connective tissue while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of ECM connective tissue as a xenograft; and

(c) sterilizing said isolated, Urodele ECM connective tissue.

21. (Original) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Currently amended) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, ~~decellularized~~ Urodele extracellular matrix (ECM) ~~or a Urodele fraction derived from the isolated, decellularized Urodele ECM~~, wherein said isolated, Urodele ECM is produced by a process comprising:

(a) obtaining a tissue sample from a Urodele, which tissue sample comprises ECM connective tissue and cells;

(b) decellularizing the sample to retain structural and functional integrity of the ECM connective tissue while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the ECM connective tissue as a xenograft; and

(c) sterilizing said isolated, Urodele ECM connective tissue.

23. (Withdrawn) A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. (Withdrawn) The tissue culture system of claim 22, wherein the cells are human cells.

25. (Withdrawn) Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. (Currently amended) A device comprising at least two sheets of isolated Urodele ~~decellularized~~ ECM laminated to one another.

27. (Withdrawn) A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. (Withdrawn) A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. (Withdrawn) The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. (Withdrawn) The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. (Withdrawn) The method of claim 30, further comprising reconstituting the powder as a gel.

32. (Withdrawn) The method of claim 29, further comprising sterilizing the decellularized ECM.

33. (Withdrawn) The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

34-35. (Canceled)

36. (Currently amended) The tissue graft of claim [[35]]5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Currently amended) The tissue graft of claim [[34]]36, wherein said process further comprises subjecting said sample to sterilization.

38. (Currently amended) The tissue graft of claim [[34]]5, wherein the Urodele is an Axolotl.

39. (Canceled)

40. (Currently amended) The sterile medical implant of claim [[39]]20, wherein the Urodele ECM includes basement membrane.

41. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM

is derived from Axolotl tissue.

42. (Currently amended) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent found in a human, whether isolated, synthetically or recombinantly produced.

43. (Previously presented) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Currently amended) The sterile medical implant of claim 20, wherein the ~~Urodel~~ Urodele ECM is ~~particles of the Urodel~~ in the form of Urodele ECM particles.

46. (Currently amended) The sterile medical implant of claim 20,[[,]] wherein the ~~Urodel~~ Urodele ECM is a reconstituted gel.

47. (Previously presented) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Previously presented) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (New) The tissue graft according to claim 5 wherein the ECM is sterile, wherein the ECM is a powder, a paste, a gel or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (New) The tissue graft of claim 49, wherein the ECM is a powder.

51. (New) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (New) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (New) The tissue graft of claim 49, wherein the ECM is a paste.

54. (New) The tissue graft of claim 49, wherein the ECM is a gel.

55. (New) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (New) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (New) The tissue graft according to claim 5 wherein the ECM is sterile, wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (New) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (New) The tissue graft of claim 57, wherein the agent is a growth factor.

60. (New) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. (New) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; at least two sheets of isolated Urodele decellularized ECM laminated to one another.

REMARKS

Applicant respectfully requests reconsideration. Claims 1-48 were previously pending in this application, with claims 1-4, 6-19, 23-25 and 27-33 being withdrawn from consideration. Claims 34, 35 and 39 have been canceled herein. Claims 5, 20, 22, 26, 36-38, 40-42, 45, and 46 have been amended herein. New claims 49-61 have been added herein. Support for the new claims is found throughout the application and claims as originally filed. As a result, claims 5, 20-22, 26, 36-38 and 40-61 are currently under examination. Applicant reserves the right to pursue the claims as previously pending, or as originally filed, in one or more continuing applications. No new matter has been added.

Claim Objections

The Examiner objected to claim 5 based on the assertion that "urodele" should be capitalized. The Examiner objected to claim 46 based on a minor typographical error. Claims 5 and 46 have been amended to capitalize the term "Urodele" and correct the typographical error, thereby rendering these objections moot.

Rejections under 35 U.S.C. § 112

The Examiner rejected claims 20, 42 and 45 under 35 U.S.C. §112 as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor regards as the invention.

The rejections to the claims under 35 U.S.C. § 112 have been addressed and overcome by amendment to the claims. Reconsideration and withdrawal of the claims on this basis is respectfully requested.

Rejection under 35 U.S.C. § 101

The Examiner rejected claims 5, 20-22, 34-41 and 44-47 under 35 U.S.C. §101 because the claimed invention is allegedly directed to a judicial exception (*i.e.*, a law of nature, a natural phenomenon, or an abstract idea) without significantly more.

First, it is noted that claims 26, 42, 43 and 48 are not rejected on this basis and are therefore acknowledged as being drawn to patent eligible subject matter. New claims 49-61 are likewise drawn to patent eligible subject matter, as those claims include similar features to those of claims 26, 42, 43 and 48 beyond the isolated, decellularized ECM *per se*.

Claims 5, 20-22, 34-41 and 44-47 are said to be "directed to an extracellular matrix (ECM), or decellularized ECM, **which are all products of nature."** Page 4 of the Office Action (emphasis added).

This is not correct. While *in vivo,* there is a structure that is referred to as "extracellular matrix", this is not the same material, morphologically or chemically, that exists *ex vivo* albeit also referred to by the term "extracellular matrix" or "ECM". Evidence to corroborate this assertion is provided and discussed herein below. For clarity, from here on out, ECM as it exists *in vivo* is referred to as ECM_{iv}, while ECM as it exists *ex vivo* is referred to herein as $ECM_{ev.}$ ECM_{iv} is a constituent part of a complex living tissue containing cells which *in vivo* are responsible for creating the ECM_{iv} within which the cells are embedded. As further discussed herein below, ECM_{ev}, as used herein, is that portion of the retained connective tissue of ECM_{iv} having been decellularized and further treated to remove cellular debris, and which exhibits vastly different properties than the connective tissue component of ECM as it existed *in vivo* as a constituent of ECM_{iv}.

It is further stated that the "claims do not include additional elements that are sufficient to amount to significantly more than the judicial exception because they read on components, such as collagen that are **not in any way significantly different than their natural counterparts in structure or function."** Page 4 of the Office Action (emphasis added).

This is not correct. The components, including collagen, that exist in ECM_{iv} and which exist in ECM_{ev} are very different from each other. Evidence to corroborate this assertion is provided and discussed herein below and in the attached Declaration of the inventor, Ryanne Early.

It is further stated that "The claims merely require such components **removed from their natural habitat, without functionally or structurally changing them.** Despite the hand of man required to effect such isolation, this alone is insufficient to materially change the individual

components that would amount to significantly more than their natural counterparts." Page 4 of the Office Action (emphasis added).

Respectfully, the Examiner's reasoning is based on a fiction, as further discussed herein below.

1. ECM_{iv} and ECM_{ev}

As discussed in the attached Declaration under 37 CFR 1.132 executed by inventor Ryanne Early, *in vivo,* ECM_{iv} performs the function of providing a structural support for cells - a scaffold, if you will, to which is covalently attached a wide range of molecules tethering cells, membranes, proteins and the like. Not only does the hand of man in this case involve removal of cellular material, but a wide array of chemical bonds are broken, and cellular material is removed, such that, biochemically, ECM_{ev} is very different from ECM_{iv}.

The Examiner has failed to establish a *prima facie* case that ECM_{ev} *per se* is a product of nature, and has further failed to establish a *prima facie* case that the ECM_{iv} and ECM_{ev} are the same material. It is certainly not the case that the ECM_{ev} contains the same absolute amounts or ratios of components that ECM_{iv} contains prior to treatment as disclosed in this application.

2. Evidence Refuting Characterization of ECM_{ev} as a Product of Nature

The present application itself contains sufficient evidence to demonstrate that ECM_{iv} and ECM_{ev}, as produced according to the present disclosure, are very different materials.

The Examiner's attention is drawn, for example, to Figures 5 and 6. FIG. 5 provides an histological evaluation of H&E-stained, paired native and post-processed sections of axolotl dermal tissue. From a gross morphological perspective, the ECM_{iv} and ECM_{ev} appear quite different, if for no other reason than that the ECM_{ev} is essentially devoid of cells. FIG. 6 presents ELISA data for DNA content in pre- and post-processed axolotl tissue, with comparison data from human amniotic membrane tissue. It is evident from this data alone that ECM_{ev} produced according to the method of this invention contains approximately 90+% less DNA than does native ECM. Given that DNA exists in the nuclei of cells and given that cells are tethered by chemical bonds to ECM *in vivo*,

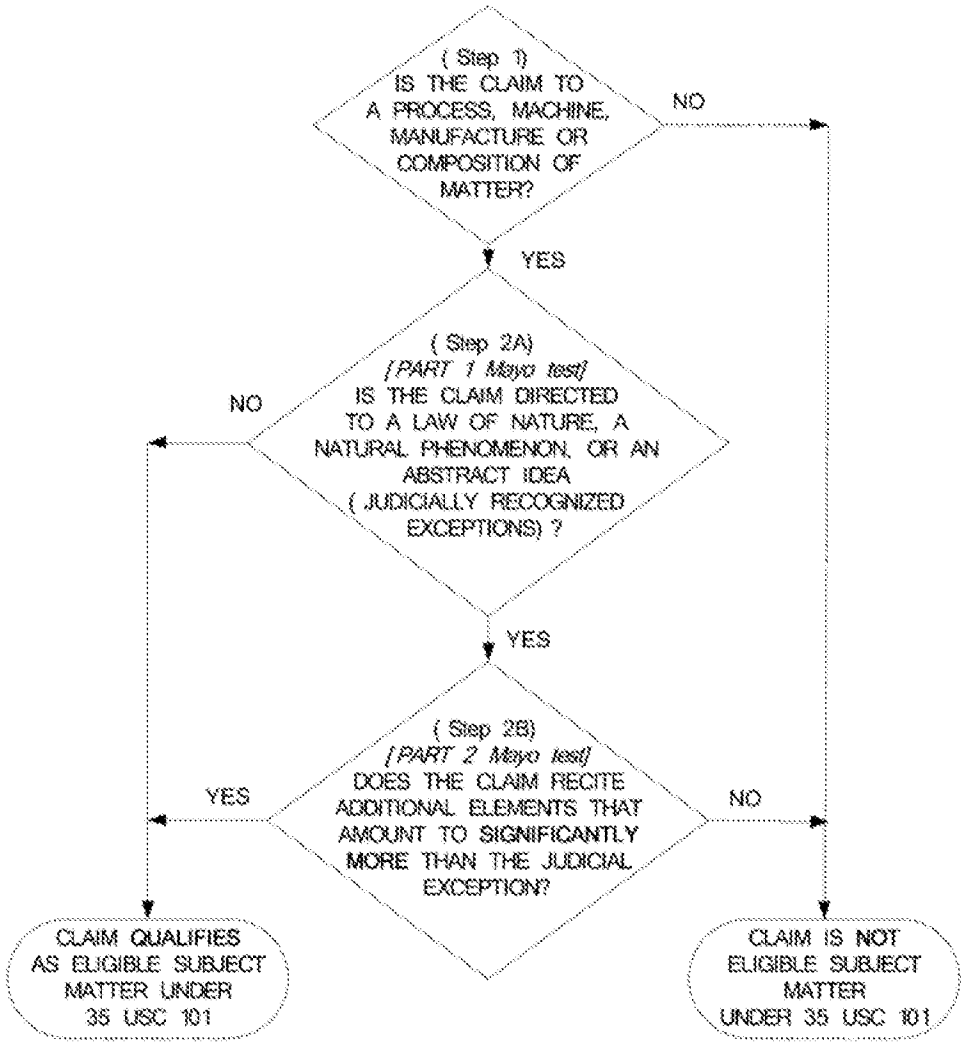
which bonds are absent from $ECM_{ev,}$ this provides conclusive evidence that ECM_{iv} and ECM_{ev} are very different chemically.

The Examiner's attention is directed to the discussion of Figure 1 in Seifert, cited by the Examiner, provided in the attached Declaration of inventor Ryanne Early. What this figure shows, via a combination of known biochemical techniques, (see the Figure 1 caption), is that ECM_{iv} is an intricate series of structures, layers, and conduits, tightly interlaced with cells, cell components, proteins, and the like. It is urged that it would simply be improper to refuse to acknowledge that ECM_{iv}, replete as it is with cells, structure and chemical bonds accommodating those cells, is in no way ECM_{ev} following one or another of a series of possible decellularization processes, since via any form of decellularization, ECM_{iv} is altered**,** and what remains is a product the nature and components of which are dictated by the process of its production, but which is chemically and functionally different from that of ECM_{iv}.

The attached Declaration by inventor Ryanne Early is in accord with and supports these assertions.

3. The Assertion that the Present Claims are not Patent Eligible Fails Step 2A (Part 1 Mayo Test), Namely: "IS THE CLAIMED INVENTION DIRECTED TO A LAW OF NATURE, A NATURAL PHENOMENON, OR AN ABSTRACT IDEA?"

As the Examiner is aware, the USPTO has issued Guidance with respect Patent Subject Matter Eligibility (see 2014 Interim Guidance; July 2015 Update; and May 2016 "Formulating a Subject Matter Eligibility Rejection and Evaluating the Applicant's Response to a Subject Matter Eligibility Rejection). Reference is made to the chart provided in the 2014 Interim Guidance. For ease of reference, the chart is reproduced below:



No law of nature has been identified in the above quoted rejection. The foregoing comments make it plain that a natural phenomenon, product of nature, or abstract idea is not what the present claims are directed to. Accordingly, Step 2A shown in the above chart is not met, which, per this chart, means that the present claims qualify as patent eligible subject matter under 35 U.S.C. § 101. Even if Step 2A is asserted to be met (which Applicant does not concede), treatment of Axolotl ECM with alkali results in the removal of cells, and production of an acellular ECM_{ev} which is isolated and stored for subsequent use as taught in the subject application. The ECM_{ev} is significantly different from the structure, function, and chemical nature and composition of ECM_{iv}. These significant differences should be recognized, in combination, to amount to significantly more

than the judicial exception asserted to be present in the claims. Reconsideration and withdrawal of this ground for rejection is therefore respectfully requested.

4. The Present Claims are Not Overbroad, Do Not Preempt Laws of Nature, and are in Accord with Current Case Law Affirming Patent Eligibility Under Section 101.

The present claims do not raise concerns of preemption. According to recent case law, the "directed to" portion of whether claimed subject matter is eligible is improper if it involves abstracting the entire claimed subject matter down to an isolated concept without consideration of all parts of the claim. A proper § 101 inquiry requires consideration of the claimed subject matter "as a whole" and in light of the teachings provided in the specification.

In combination, the recent US Supreme Court decisions in Association for Molecular Pathology v. Myriad Genetics, 133 S. Ct. 2107, and Mayo v. Prometheus, 132 S. Ct. 1289 (2012), have resulted in the conclusion that claims directed to isolated genomic DNA (gDNA), removed and separated from its natural environment in the cell, is not patentable. However, it has been confirmed that claims directed to cDNA are patent eligible, because cDNA has been recognized as not existing in nature ("synthetically created DNA...which contains the same protein-coding information found in a segment of natural DNA but omits portions within the DNA segment that do not code for proteins"). That is to say, cDNA was found to be chemically different than sequences found in natural DNA. By analogy, merely removing ECM_{iv} blood from a living Urodele would not be sufficient to render the ECM_{iv} patent eligible. However, ECM_{ev}, which is chemically different than ECM_{iv} as found in nature, is analogous to cDNA, and should be found patent eligible. Therefore, provided it meets the other requirements of the patent statute, it would be consistent with US Supreme Court precedent to hold that the composition claimed herein is patent eligible.

The Supreme Court has since clarified, in Alice Corp. V. CLS Bank International, 134 Ct. 2347 (2014), that the two-part test under 35 USC § 101 as previously established in Mayo, includes determining whether the claims contain a patent ineligible concept, and if so, determining whether the patent claim embodies an "inventive concept." It is urged that the present claims, even if considered to reference a patent ineligible concept, (*e.g.*, ECM_{iv} –a tissue sample), embodies the inventive concept of producing ECM_{ev} by the disclosed decellularization process to ensure that the

resulting ECM_{ev} has significantly different properties when used to treat a vertebrate than would ECM_{iv}. The latter would be rejected as a foreign body when introduced, for example, as a wound dressing in a human, whereas ECM_{ev} produces rapid healing and is not rejected.

The Court of Appeals for the Federal Circuit, in Enfish, LLC v. Microsoft Corp, 822 F.3d 1327 (2016), has explained that the common "directed-to" practice of invalidating claims based on the Mayo/Alice inquiry is wrong, and that the character of the claimed invention as a whole (including all parts of the claim) must be used to conduct the eligibility inquiry. Thus, the Court implied/explained in pertinent part that a claim can be patent eligible if the known procedures as a whole are not unduly preempting or too obvious when considering the advancement of the invention. In the present case, bearing in mind this jurisprudence, it cannot properly be maintained that the present claims are "directed to" a patent-ineligible concept, natural phenomenon or product of nature, and the significant advancement provided by the invention is one that has huge therapeutic utility.

"In setting up the two-stage Mayo/Alice inquiry, the Supreme Court has declared: "We must first determine whether the claims at issue are directed to a patent-ineligible concept." Alice, 134 S. Ct. at 2355. That formulation plainly contemplates that the first step of the inquiry is a meaningful one. The "directed to" inquiry, therefore, cannot simply ask whether the claims involve a patent-ineligible concept, because, at some level, essentially every routinely patent-eligible claim involving physical products and actions involves a law of nature and/or natural phenomenon. See Mayo, 132 S. Ct. at 1293 ("For all inventions at some level embody, use, reflect, rest upon, or apply laws of nature, natural phenomena, or abstract ideas."). Rather, the "directed to" inquiry applies a stage-one filter to claims, considered in light of the specification, based on whether "their character as a whole is directed to excluded subject matter." See Genetic Techs. Ltd. v. Merial L.L.C., 2016 WL 1393573, at *5 (Fed. Cir. 2016) (inquiring into "the focus of the claimed advance over the prior art").

Likewise, in Bascom v. AT&T, No. 2015-1763, the Federal Circuit reversed a lower court's § 101 invalidity finding because the lower court's ruling was based on the limitations of the claims which, taken individually, showed nothing new as would be needed to prevent an otherwise abstract/preemptive invention from being patent eligible. While both Bascom and Enfish were

directed to software, the statute, the case law, and the applicable patent-eligibility test for the analysis include common and closely-linked analyses, including concerns whether the claims are so broad as to preempt laws of nature. Thus, in Rapid Litig. Mgt. v. CellzDirect, Inc., No. 2015-1570 (CAFC, July 5, 2016), the Court upheld patent claims directed to ways of preserving liver tissue (hepatocyte cells) by using multiple freeze-thaw cycles. Applying the Mayo/Alice test, the Federal Circuit clarified and distinguished the patent claims from Mayo, Alice, and Sequenom, explaining that the claims were "not directed to a patent ineligible concept" (*i.e.*, the ability of hepatocyte cells to survive multiple freezes), but to a new and useful laboratory technique for preserving hepatocytes. Specifically, the Federal Circuit indicated that the "inventors certainly discovered the cells' ability to survive, but that is not where they stopped, nor is it what is patented." The Federal Circuit further indicated that while pre-emption is not the test for determining patentability, the claims at issue did not lock up the law of nature in its entirety.

Along these same lines, the Federal Circuit, in McRO, Inc. v. Bandai Namco Games America Inc., Docket Nos. 2015-1080 (Fed. Cir. Sept. 13, 2016), further clarified the preemption concern and indicated that where the claimed invention is limited to specific, unconventional steps, the "directed to" application of §101 fails. The Federal Circuit upheld the patent claims, explaining that "[c]laims to the genus of an invention, rather than a particular species, have long been acknowledged as patentable....The preemption concern arises when the claims are not directed to a specific invention and instead improperly monopolize 'the basic tools of scientific and technological work.' Alice, 134 S. Ct. at 2354 (quoting Myriad, 133 S. Ct. at 2116). The abstract idea exception has been applied to prevent patenting of claims that abstractly cover results where 'it matters not by what process or machinery the result is accomplished.'

Consistent with this jurisprudence, the present claims are carefully directed to a non-natural composition of matter which is different in constituents, manner of production, and beneficial uses as compared to ECM_{iv} which occurs in nature. Accordingly, it is respectfully urged that the rejection based on patent ineligibility under 35 USC § 101 should be reconsidered and withdrawn.

Rejections under 35 U.S.C. § 102

The Examiner rejected claims 5, 20-22, 34-37, 45 and 46 under 35 U.S.C. § 102 as being anticipated Smith et al. (1982). It is asserted that "Smith teaches compositions comprising collagen (ECM components, decellularized ECM or fractions thereof) from Ambystoma tigrinum (a Urodele) (abstract, entire reference). Although Smith does not teach the process by which the collagen is produced, these limitations are considered to product by process type limitations. It is noted that the patentability of a product does not depend on its method of production, If the claimed product is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference), the claim is unpatentable even though the reference product was made by a different process. When the prior art discloses a product which reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C 102 and/or 35 USC 103 are proper, (MPEP2113). Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen. Therefore, the reference anticipates the claimed subject matter." Pages 4-5 of the Office Action. Reconsideration is requested.

In fact, Smith teaches nothing whatever about ECM *per se,* i.e. neither ECM*iv* nor ECM*ev* is taught or suggested by Smith. A review of the Materials and Methods section of Smith, page 244, left hand column, corroborates this assertion. There it is disclosed that the *Ambystoma* is decapitated "and the skins and cartilages dissected from other tissues and placed on ice." Note, there is no decellularization of the tissue. Accordingly, this still only comprises ECM*iv*. The "skins were subsequently extracted in 0.4 ionic strength phosphate buffer, pH 7.6, and three changes of 0.5 M **acetic acid.** Each extract was clarified by centrifugation, and **the collagen in the supernatant was purified**".

Thus, per Smith, an acid soluble fraction of collagen is isolated, purified and characterized. There is no isolation, purification, or characterization of ECM. Per the described process of Smith, any ECM present in the starting material would be found as a pellet in the centrifugation process, along with any other acid insoluble debris, such as cellular components and the like, with proteins such as collagens extracted into the supernatant. Accordingly, any ECM that existed in the starting

material of Smith is actually **completely destroyed** and discarded by the extraction that Smith conducts. Reconsideration and withdrawal of this ground for rejection is respectfully requested.

The Examiner rejected claims 5, 20-22, 34-39, 41, 44-47 under 35 U.S.C. § 102 as being anticipated Satoh et al. (2012). It is alleged that "Satoh teaches acellular collagen sheets (ECM components, decellularized ECM or fractions thereof) from axolotl (p192). **Although Satoh does not teach each of the claimed process steps by which the collagen is produced, these limitations are considered to product by process type limitations**. It is noted that the patentability of a product does not depend on its method of production, **if the claimed product is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference**), the claim is unpatentable **even though the reference product was made by a different process,** When the prior art discloses a product which **reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C. 102 and/or 35 U.S.C. 103 are proper**, (MPEP § 2113). Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen. Therefore, the reference anticipates the claimed subject matter." Pages 5-6 of the Office Action (emphasis added). Reconsideration is requested.

A correct reading of Satoh reveals the following: At page 192, bottom of left hand column to the top of the right hand column, Satoh provides the following description of how an "acellular collagen sheet" was produced from Axolotl tissue:

> The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, **and then incubated in water with 0.01% Tween 20, PMSF (1.74 μg/ml), and glutaraldehyde** (20.18 μg/ml) for two days, resulting in an acellular ECM sheet. The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 3rC (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed on the rerouted nerve. (emphasis added)

Accordingly, Satoh disclose only the production of a miniscule amount of ECM obtained from an axolotl (i.e. skin from the Axolotl upper limb). In fact, however, the process disclosed according to Satoh fails to meet the requirement in the present claims of a decullularizing process comprising: "(i) retaining the structural and functional integrity of said tissue sample; and (ii)

removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft."

Per the above-quoted process of Satoh, what in fact was produced a cross-linked matrix (due to the glutaraldehyde) which would not retain the structural and function integrity of the tissue sample. In addition, by cross-linking the material produced according to Satoh, both the cross linking itself and the fact that such cross linking would covalently bind any cellular debris still present (since Satoh teaches treatment of the entire native ECM with detergent to rupture cells and no separation of such material is taught prior to glutaraldehyde treatment) fails to meet the requirement in the present claims that sufficient cellular components are removed to reduce or eliminate antigenicity of the biomaterial as a xenograft, since glutaraldehyde treatment of proteins generally increases antigenicity. (See the Declaration of Ryanne Early). In fact, reducing antigenicity was not even a goal in Satoh, because the cross-linked matrix was syngeneic with the animal to which the matrix was applied.

Accordingly, withdrawal of this rejection is respectfully requested.

The Examiner rejected claims 5, 22 and 47 under 35 U.S.C. § 102 as being anticipated Seifert al. (2012). It is asserted that "Seifert teaches an ECM from axolotl. Regarding claim 22, the various skin samples disclosed therein are considered materials impregnated with, coated with or encapsulating, or having attached thereto, Urodele ECM. Therefore, the reference anticipates the claimed subject matter." Page 6 of the Office Action. Reconsideration is requested.

While Seifert mentions ECM and extracellular matrix throughout, this is all in the context of *in vivo* modelling of ECM production as it relates to scar tissue formation. In fact, a thorough review of Seifert has failed to reveal any disclosure of production of anything like an ECM_{ev}. The closest that has been found to any such disclosure in Seifert is found on page 17 which provides:

> For histological and immunohistochemical analysis, **harvested tissues were fixed in 10% NBF** at 4 °C for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4 °C and **processed for paraffin embedding**. Samples were cut at 5mm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4 °C, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT. (emphasis added)

According to Dr. Early, "NBF is Neutral Buffered Formalin, or formaldehyde, a cross-linking agent. If the Examiner is asserting that Seifert's histological section preparation steps of fixing and embedding a tissue specimen and the product produced anticipates a process used to prepare tissue product and the product produced which is suitable for implantation, then it is my opinion that such assertion is simply illogical. Accordingly, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of retaining structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft." Declaration of Ryanne Early, ¶6.

Accordingly, withdrawal of this rejection is respectfully requested.

Rejections Under 35 U.S.C. § 103

The Examiner rejected claims 5, 20-22, 26 and 34-48 under 35 U.S.C. § 103(a) as being unpatentable over Asem et al. (US6485969), in view of Seifert et al. (2012). It is stated that "Asem teaches decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40). The ECM may in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45)." Pages 6-7 of the Office Action.

It is gratefully acknowledged that the Examiner concedes that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, it is asserted that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful

for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious." Page 7 of the Office Action. Reconsideration is requested.

The combination proposed by the Examiner in rejecting the claims is not based on sound reasoning as required under the law. It is believed that the combination proposed by the Examiner does not make sense and is made only with hindsight, after study of the present application, in order to reject the claims.

The Examiner has conceded that Asem does not teach the ECM wherein the amphibian is a Urodele or axolotl. To address this deficit in Asem, the Examiner combines the Asem teaching with that of Seifert. However, as noted above in response to the anticipation rejection based on Seifert, the only mention of ECM in Seifert is in the context of healing ECM *in vivo* as it relates to scar-free tissue regeneration. Further, as noted above, it is simply illogical to assert that Seifert's teaching of histological section preparation steps for fixing and embedding a tissue specimen and the product produced by such a method anticipates, teaches or suggests a process to prepare tissue product and the product produced which is suitable for implantation as a xenograft. Seifert neither teaches nor suggests the present process or product and, those skilled in the art aware of Asem would only have found in Seifert a study of the wound healing processes in the Axolotl. In fact, Seifert teaches that one would want to avoid or delay the presence of ECM in promoting scar-free healing, teaching away from the combination suggested by the Examiner. For these reasons, the prior art does not teach or suggest the claimed invention, that is, a sterile, isolated, decellularized Urodele extra cellular matrix (ECM) produced by a process comprising "(A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft."

Accordingly, Seifert fails to cure the deficits in Asem noted above, for the reasons already noted in Seifert alone as an anticipatory reference. Reconsideration and withdrawal of this ground for rejection is respectfully requested.

New Claims

With respect to the new claims introduced into this application by this amendment, it is respectfully urged that the arguments and remarks provided herein above with respect to claims 5, 20, 22, 26 and 36-48, apply to the new claims *a fortiori*. Furthermore, as claims 49-56 are directed to tissue grafts that are in the form of powders pastes, gels, sheets and mesh, it is urged that these claims meet the requirements of section 101, based on the USPTO "Subject Matter Eligibility Examples: Life Sciences."

The powder limitation of the present claims is similar to that in Example 30, claim 5 of the USPTO Examples. Referring to Example 30, claim 5:

> 5. A dietary sweetener comprising: granular particles of texiol having a particle diameter of X10 of 80 microns and X90 of 300 microns.

It is stated about this claim:

> This altered property is a marked difference in characteristics, because it results in the claimed formulation being distinct from its natural counterpart in a way (release of sweetness over time) that is relevant to the nature of the invention as a dietary sweetener, e.g., because the claimed formulation will dissolve evenly and rapidly in a cool liquid. Cf. Roslin, 750 F.3d at 1339 (claimed cloned mammals do not have markedly different characteristics from their naturally occurring counterparts). Because it has markedly different characteristics as compared to its natural counterpart, the claimed formulation is not a "product of nature" exception. Thus, the claim is not directed to an exception (Step 2A: NO), and qualifies as eligible subject matter.

In the present application, new claim 49, to the extent it is limited to a powder, is aligned with Example 30, claim 5, and is patent eligible. The closest naturally occurring component is the ECM, which is a wet layer *in vivo* of extracellular matrix infiltrated with cells. It has a macroscopic shape that conforms to body tissue. The powder as claimed, on the other hand, has only a microscopic dimensionality and is useful, for example, to reconstitute a gel or a paste of the ECM, which can in turn be used as a tissue graft to form particular shapes, to completely fill openings without voids, to spray tissue graft materials, to mention only some of its beneficial uses. Because

the claimed powder has markedly different characteristics as compared to its natural counterpart, the claimed formulation does not fit the "product of nature" exception. Thus, the claim is not directed to an exception (Step 2A: NO), and qualifies as eligible subject matter.

The same can be said of the paste, the gel, and the mesh. The claimed paste, gel and mesh have markedly different characteristics as compared to their natural counterpart, and the claimed formulation does not fit the "product of nature" exception. Thus, the claim is not directed to an exception (Step 2A: NO), and qualifies as eligible subject matter.

Example 28, claim 4 of the USPTO guidelines, also provides similar guidance.

> 4. A vaccine comprising: Peptide F; and a pharmaceutically acceptable carrier selected from the group consisting of a cream, emulsion, gel, liposome, nanoparticle, or ointment.

In this example, the natural product Peptide F was combined with a carrier to form a product with different characteristics from the natural product. In essence, it worked better as an immunogen. It was stated:

> The cream's changed form and adherence are marked differences in structural and physical characteristics as compared to the natural counterparts, and therefore the cream is not a "product of nature" exception. Thus, the claim is not directed to an exception (Step 2A: NO), and qualifies as eligible subject matter.

In the case of the claimed powder, paste, gel or mesh, the 'changed form and adherence are marked differences in structural and physical characteristics as compared to the natural counterparts, and therefore … not a "product of nature" exception'.

Likewise, new claims 49-56 are not anticipated by Smith et al. Smith et al. teach isolation of collagen from ECM. Smith et al. wanted to characterize the collagen in ECM. The claims are now directed to decellularized ECM, not also an isolate from decellularized ECM. The claimed product is in the form of a powder, a paste, a gel or a mesh. Smith et al. do not teach or suggest a product that is decellularized ECM in the form of a powder, a paste, a gel or a mesh. Smith et al. teach only a collagen extract of ECM. Decellularized ECM, whether a powder, paste, gel or mesh, has a three-

dimensional structure and contains more than collagen. The claims, therefore, are not anticipated by Smith et al.

Claims 49-56 are not anticipated by Satoh et al. Satoh et al. teach the use of a sheet of glutaraldehyde treated, decellularized ECM. Satoh et al use this material in experiments investigating the effects of collagen deposition on limb regeneration, with a particular focus on demonstrating that collagen can inhibit the communication between nerve cells and regenerative cells, which communication is necessary for limb regeneration. Satoh et al. do not teach or suggest a powder, a paste, a gel or a mesh that comprises decellularized ECM. Satoh only teach a sheet of glutaraldehyde treated ECM. The claims, therefore, are not anticipated by Smith et al.

Claims 49-56 are not anticipated by Seifert et al. Seifert et al. teach part of the mechanism for scar-free healing in Axolotls. Seifert et al. teach "a relatively long delay in production of new extra cellular matrix (ECM) in scar free healing." In other words, Seifert et al. teach that scar-free healing is associated with the delayed deposition of ECM as compared to the timing of deposition of ECM in scar-associated healing, e.g. in vertebrates. Seifert et al. do not teach decellularized ECM in any form in which structure and function are retained, let alone in the form of a powder, a paste, a gel or a mesh.

Claims 49-56 are not obvious over Asem in view of Seifert et al. Asem teaches the use of follicular ECM from vertebrates, particularly those that lay eggs. Asem teaches follicular tissue can be a substitute for existing products made from placental tissue. Asem does not suggest using any ECM other than follicular ECM.

Seifert et al. teach "a relatively long delay in production of new extra cellular matrix (ECM) in scar free healing. Seifert et al., if anything, suggests delaying ECM production if the desire is for scar free healing.

The same reasons as provided herein above as to why claims 5, 20-22, 26 and 34-48 are not rendered obvious by the combination of Asem and Seifert apply to new claims 49-56 *a fortiori*, as, in addition, these claims are directed to ECM_{ev} in the form of a powder, a paste, a gel or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM. This is neither taught nor suggested by Asem alone, Seifert alone, or by the combination thereof.

With respect to new claims 57-61, it is urged that these claims meet the requirements of section 101, based on the USPTO "Subject Matter Eligibility Examples: Life Sciences." The Examiner's attention is directed to the discussion of Example 30, claim 5 and Example 28, claim 4, discussed above.

Claims 57-61 require that the ECM be in the form of a sheet. A sheet of decellularized ECM does not occur in nature. Instead, in nature, the ECM conforms to the shape of the Urodele's body. As such, it is irregular and not useful, for example, to treat or prevent disease, damaged, weakened or ruptured tissue or a wound. It is immunogenic as it occurs in nature, and is thus not useful as a universal tissue graft. In addition, in nature, the Urodele ECM sheet is not infused with, coated with, or attached to an agent xenogeneic to a Urodele. Depending on the agent combined with the sheet, the sheet can be fashioned to be applied to a wound, fashioned to deliver a drug, an antimicrobial agent, and so on. These changes are marked differences in structural and physical characteristics as compared to the natural counterpart, and therefore the claimed sheet is not directed to an exception, and qualifies as eligible subject matter.

Claims 57-61 are not anticipated by Smith et al. Smith et al. teach isolation of collagen from ECM. Smith et al. wanted to characterize the collagen in ECM. The claims are now directed to decellularized ECM, not also an isolate from ECM, in the form of a sheet. Smith et al. do not teach or suggest a decellularized ECM sheet, which is a three-dimensional structure, not a molecular isolate. Smith does not teach combining an ECM decellularized sheet with an agent xenogeneic to a Urodele. The claims, therefore, are not anticipated by Smith et al.

Claims 57-61 are not anticipated by Satoh et al. Satoh et al. teach the use of a sheet of glutaraldehyde treated, decellularized ECM. Satoh et al use this material in experiments investigating the effects of collagen deposition on limb regeneration in a Urodele, with a particular focus on demonstrating that collagen can inhibit the communication between nerve cells and regenerative cells, which communication is necessary for limb regeneration. Satoh et al. do not teach or suggest a combining an ECM decellularized sheet with an agent xenogeneic to ta Urodele, wherein the agent is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug. Satoh only teach a

sheet of glutaraldehyde treated, decellularized ECM. The claims, therefore, are not anticipated by Smith et al.

Claims 57-61 are not anticipated by Seifert et al. Seifert et al. teach part of the mechanism for scar-free healing in Axolotls. Seifert et al. teach "a relatively long delay in production of new extra cellular matrix (ECM) in scar free healing." In other words, Seifert et al. teach that scar-free healing is associated with the delayed production of ECM. Seifert et al. do not teach decellularized ECM in a form which retains structure and function while being non-antigenic as a xenograft, as required by the present claims.

Claims 57-61 are not obvious over Asem in view of Seifert et al. Asem teaches the use of follicular ECM from vertebrates, particularly those that lay eggs. Asem teaches follicular tissue can be a substitute for existing products made from placental tissue. Asem does not suggest using any ECM other than follicular ECM.

Seifert et al. teach "a relatively long delay in production of new extra cellular matrix (ECM) in scar free healing." In other words, Seifert et al. teach that scar-free healing is associated with the *absence* of ECM. Seifert et al., if anything, suggests avoiding production or presence of ECM if the desire is for scar free healing.

The same reasons as provided herein above as to why claims 5, 20-22, 26 and 34-48 are not rendered obvious by the combination of Asem and Seifert apply to new claims 57-61 *a fortiori*, as, in addition, these claims are directed to ECM_{ev} in the form of a sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; wherein the agent is an anti-microbial agent;, wherein the agent is a growth factor; wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another; or wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or

attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; at least two sheets of isolated Urodele decellularized ECM laminated to one another.

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Page 657

CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, the Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 23/2825, under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Dated: July 25, 2017

Respectfully submitted,

By: /Edward R. Gates/
Edward R. Gates
Registration No.: 31,616
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Electronic Patent Application Fee Transmittal

Application Number:	14759895
Filing Date:	08-Jul-2015
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Filer:	Edward R. Gates/Eileen MacKenzie
Attorney Docket Number:	S1860.70000US01

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	2253	1	700	700
Miscellaneous:				
			Total in USD ($)	**700**

Electronic Acknowledgement Receipt

EFS ID:	29889046
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Eileen MacKenzie
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	25-JUL-2017
Filing Date:	08-JUL-2015
Time Stamp:	19:38:39
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$700
RAM confirmation Number	072617INTEFSW19392500
Deposit Account	232825
Authorized User	Wolf Greenfield

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	S186070000US01-TRN-ERG.pdf	25394 773af5b43cc5882cb9d6736e03078ac330103803	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	S186070000US01-FEE-ERG.pdf	31403 24f5199d3554bee92f129d07af12d2b205575c38	no	1
Warnings:					
Information:					
3	Extension of Time	S186070000US01-EXT-ERG.pdf	23557 2253435c717b049bd73b4efa5e2f23105d7c6d5f	no	1
Warnings:					
Information:					
4		S186070000US01-AMN-ERG.pdf	191561 a50ae50ddfe99a8e0f43c4f8a87f7c252ebf1b77	yes	29

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	9
Applicant Arguments/Remarks Made in an Amendment	10	29

Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30833 5befeb3b8c000131bbbb124064fcea580bca8c82	no	2

Warnings:	
Information:	
Total Files Size (in bytes):	302748

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	R. A. Davis
Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

- [X] Fee Transmittal Form
  - [ ] Fee Attached
- [X] Amendment/Reply
  - [ ] After Final
  - [X] Affidavits/declaration(s)
- [X] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [ ] Other Enclosure(s) (please Identify below): Ryanne Early CV

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Edward R. Gates/		
Printed name	Edward R. Gates		
Date	July 25, 2017	Reg. No.	31,616

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: July 25, 2017 Electronic Signature for: /Eileen M. MacKenzie/

5609643.1

FEE TRANSMITTAL

Complete if known	
Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Examiner Name	R. A. Davis
Art Unit	1651
Practitioner Docket No.	S1860.70000US01

[X] Applicant asserts small entity status. See 37 CFR 1.27.

[ ] Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)700.00

METHOD OF PAYMENT (check all that apply)

[ ] Check [X] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[ ] Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

[ ] Charge fee(s) indicated below [ ] Charge fee(s) indicated below, **except for the filing fee**

[X] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 [X] Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	280	140*	70	600	300	150	720	360	180	
Design	180	90	45	120	60	30	460	230	115	
Plant	180	90	45	380	190	95	580	290	145	
Reissue	280	140	70	600	300	150	2,160	1,080	540	
Provisional	260	130	65	0	0	0	0	0	0	

* The $140 small entity status filing fee for a utility application is further reduced to $70 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	80	40	20
Each independent claim over 3 (including Reissues)	420	210	105
Multiple dependent claims	780	390	195

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
0	- 20 or HP =	0		x =	

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
0	- 3 or HP =	0		x =	

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
	- 100 =		/50 =	(round **up** to a whole number) x	=	

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 2253 Extension for response within third month	700.00

SUBMITTED BY

Signature	/Edward R. Gates/	Registration No. (Attorney/Agent)	31,616	Telephone	617.646.8000
Name (Print/Type)	Edward R. Gates			Date	July 25, 2017

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: July 25, 2017 Electronic Signature for: /Eileen M. MacKenzie/

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) S1860.70000US01
Application Number 14/759,895-Conf. #3358	Filed July 8, 2015
For DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	
Art Unit 1651	Examiner R. A. Davis

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☒	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 700.00
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☒ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

☐ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 31,616 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Edward R. Gates/ — Signature
July 25, 2017 — Date

Edward R. Gates — Typed or printed name
617.646.8000 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: July 25, 2017 Electronic Signature for: /Eileen M. MacKenzie/

5609645.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; **371 date: Jul. 8, 2015** : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

INVENTOR'S DECLARATION UNDER 37 C.F.R. 1.132
TO ACCOMPANY THE RESPONSE, UNDER 37 C.F.R. 111, TO NON-FINAL OFFICE ACTION, MAILED ON 01/25/2017

TO: THE COMMISSIONER OF PATENTS:
& EXAMINER DAVIS

1. I hereby declare that I am the same Ryanne Early named as a sole inventor in the subject pending patent application, and whose CV is attached to this Declaration.

2. I am aware that the Examiner in the subject application, in an Office Action dated 1/25/2017, has rejected all pending claims as being a product of nature, as being anticipated by cited art and as obvious in light of a combination of cited art. I am also aware of and have read the Amendments to the claims and arguments in support of patentability that are being filed concurrently with this Declaration.

3. It is my opinion that it is incorrect that the claims in the subject application are directed to a law of nature or to a natural product. *In vivo,* there is a structure that is referred to as "extracellular matrix", (ECM) which is not the same material, morphologically, physicochemically, biochemically, exhibiting different surface chemistry interaction potential and functionality as compared with that which exists *ex vivo*, albeit also referred to by the term "extracellular matrix" or "ECM". For clarity, adopting the terminology used in the attached

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Page 667

Amendment, ECM as it exists *in vivo* is referred to as ECM_{iv}, while ECM as it exists *ex vivo* is referred to herein as ECM_{ev}.

ECM_{iv} performs the function of providing a structural support for cells - a scaffold of connective tissue to which is attached (via, e.g. integrins and/or other attachments) a wide range of molecules, cells, membranes, proteins and the like. The ECM_{ev} according to this invention has been denuded of intact cellular material. In addition, a wide array of chemical bonds are broken, cellular material is removed, and biochemical constituents changed, such that ECM_{ev} is, in fact, very different from ECM_{iv}.

FIG. 5 in the subject application provides a histological evaluation of H&E-stained, paired native and post-processed sections of axolotl dermal tissue. Clearly, even from just a gross morphological perspective, the ECM_{iv} and ECM_{ev} are quite different, if for no other reason than that the ECM_{ev} is essentially devoid of cells.

FIG. 6 presents ELISA data for DNA content in pre- and post-processed axolotl tissue, with comparison data from human amniotic membrane tissue. It is evident from this data alone that ECM_{ev} produced according to the method of this invention contains approximately 90+% less DNA than does native ECM. This demonstrates that intact cells and cellular debris has been removed, providing a porous structure that is now conductive and can support ingrowth, which is not the case for native ECM. Furthermore, the density, deformation, and absorption characteristics of ECM_{ev} differs significantly to that of native ECM_{iv}.

If ECM_{iv} were to be analogized with genomic DNA, it is noted that while genomic DNA *in vivo* can exist in excised forms, ECM tissue does not exist in the body in an excised form. That is, *in vivo*, it would be an oxymoron to refer to "decellularized extracellular matrix", as ECM is laid down by cells, at appropriate developmental stages, by the cells that form it and in which they themselves are integral. Accordingly, formation of ECM_{ev} from ECM_{iv} is analogous to the chemical and physical transformation required to produce a cDNA.

What is clear from the data presented in Figures 5 and 6 in the present patent disclosure is that ECM_{iv} is an intricate series of structures, layers, and conduits, tightly interlaced with cells, which

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contain DNA), cell components, proteins, and the like. ECM_{iv}, replete as it is with cells and structure accommodating those cells, is in no way the same as ECM_{ev} following the decellularization process taught in the subject patent disclosure. Per this disclosure, ECM as it exists *in vivo*, i.e. ECM_{iv}, is isolated, extraneous molecules, cells and tissue is removed, to produce Urodele connective tissue, (synonymous herein with ECM_{ev}), characterized by retention of structural and functional integrity coupled with reduced or eliminated antigenicity, due to removal of sufficient cellular components to achieve that characteristic. Nothing like this is disclosed, taught or suggested in Seifert.

While intact native tissue, as shown in Seifert, is a composite material consisting of cells (interstitial and basement membrane), Extra Cellular Matrix (fibrous protein, non-fibrous protein, and glycosaminoglycans), and ground substance, the biomaterial claimed in the present application has been found to exhibit beneficial bioinductive and bioconductive qualities, as compared with native tissue in which cells preoccupy adhesion sites, exhibit minimum binding affinity, and exhibit such antigenicity as to be unusable as a xenograft.

Transplanar discontinuity, which is evident only in excised vs. intact tissue, exhibits a fracture surface of ECM which is otherwise continuous in native tissue. This fracture surface has additional adhesion sites and permits for ingrowth into pores in the matrix vs. solely via facial surface in native tissue, and exhibits altered pore kinetics. This contributes to altered reaction rates ordinarily observed only in native tissue restricted to facial surface access, mass transport and diffusion. It also contributes to altered stability since the opportunity and rate of hydrolysis additionally increases.

Native soft connective tissue exhibits trans-planar continuity, existing in a state of tensintegrity, that is concurrent radial compression and tension. This leads to physical geometry indicative of any material under such stresses: deformation, reduced thickness, and tensioning of interwoven fibers. Because these features further impact on resultant porosity, pore kinetics, and masked or available adhesion sites, additional contributions to structural and function properties occur. However, once native tissue has been clarified or decellularized, the remaining biomaterial from decellurallized urodele tissue is formed into three-dimensional shapes as desired. It is optimized for binding pharmaceutical agents, for subsequent efficient secondary processing, including

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solubilzation and covalent tethering, and loading, which cannot be done with native tissue due to challenges caused by cellular material or other impurities.

The biomaterial comprised of optimized isolated and decellularized ECM according to this invention additionally is signifiacntly more thermally stable becfause of its lack free H_20, and because of the ECM exposure to divalent oxygen there is no way to effectively eliminate the generation of free radicals within the biomaterial. Absent these key feartures, there would exist limited ability to subject the biomaterial to ionizing radiation as is done in terminal sterilization, because high moiusture native tissue would in the same condition undergo signifaicnt alteration involving concurrently the disruption of native covalent bonds in and between ECM and cells. In addition, new unnatural covalent bonds in and between ECM and cellular materials would be formed because of the high variability and lack of normal distriburtion of properties and consituents within and between tissues of the same and different animals. There is no practical means to implement use of terminal sterilization in native tissue beause of the inconsistency of imparted unique atlerations which would result even when all controllable parameters are maintained with tight tolerances. The same exact challenges present for lyphoization based secondary processing where varying celular impurities impair the ability to prepare formed shapes of material using freeze drying.

4. I am aware that claim(s) 5, 20-22, 34-37, 45 and 46 are rejected under 35 USC 102al as being anticipated by Smith et a1. (1982).

It is my opinion that Smith teaches nothing whatever about ECM *per se,* i.e. neither ECM*iv* nor ECM*ev* is taught or suggested by Smith. A review of the Materials and Methods section of Smith, page 244, left hand column, corroborates this assertion. There it is disclosed that the *Ambystoma* is decapitated "and the skins and cartilages dissected from other tissues and placed on ice." Note, there is no decellularization of the tissue. Accordingly, this still only comprises ECM*iv*. The "skins were subsequently extracted in 0.4 ionic strength phosphate buffer, pH 7.6, and three changes of 0.5 M **acetic acid.** Each extract was clarified by centrifugation, and **the collagen in the supernatant was purified**".

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Thus, per Smith, an acid soluble fraction of collagen is isolated, purified and characterized. There is no isolation, purification, or characterization of ECM. Per the described process of Smith, any ECM present in the starting material would be found as a pellet in the centrifugation process, along with any other acid insoluble debris, such as cellular components and the like, with proteins such as collagens extracted into the supernatant. Accordingly, any ECM that existed in the starting material of Smith is actually **completely destroyed** and discarded by the extraction that Smith conducts. Reconsideration and withdrawal of this ground for rejection is respectfully requested.

5. I am further aware that Claims 5, 20 - 22, 34 - 39, 41, 44 — 47 are rejected under 35 U.S.C. 102al as being anticipated by Satoh et a1, (2012).

It is my opinion that Satoh does not teach an ECM_{ev} product that is the same or even similar to the presently claimed ECM_{ev}. At page 192, bottom of left hand column to the top of the right hand column, Satoh provides the following description of how an "acellular collagen sheet" was produced from Axolotl tissue:

"The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, **and then incubated in water with 0.01% Tween 20, PMSF (1.74 µg/ml), and glutaraldehyde** (20.18 µg/ml) for two days, resulting in an acellular ECM sheet. The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 3°C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed on the rerouted nerve."

Accordingly, Satoh does not disclose production of a material for use as a xenograft - the Satoh material is used on an Axolotl. Satoh also only disclose the production of a miniscule amount (due to the tiny amount of skin available from the Axolotl upper limb) of "an acellular ECM sheet". Furthermore, the Satoh material is obtained from an axolotl in the form of a cross-linked matrix (due to the glutaraldehyde) which would not retain the structural and functional integrity of the tissue sample. In addition, by cross-linking the material produced according to Satoh, both

the cross linking itself and the fact that such cross linking would covalently bind any cellular debris still present (since Satoh teaches treatment of the entire native ECM with detergent to rupture cells and no separation of such material is taught prior to glutaraldehyde treatment), the Satoh material fails to meet the requirement in the present claims that sufficient cellular components are removed to reduce or eliminate antigenicity of the biomaterial as a xenograft. Per Satoh, this cannot be achieved due to the covalent cross-linking of cellular debris to the connective tissue matrix. Furthermore, glutaraldehyde treatment of proteins and cellular extracts generally increases antigenicity.

By contrast, the product according to the present invention is produced by a process comprising decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft. Accordingly, Satoh does not anticipate the present claims.

6. I am aware that claims 5, 22 and 47 are rejected under 35 USC, 102a1 as allegedly being anticipated by Seifert et al., (2012).

It is my opinion that while Seifert mentions ECM and extracellular matrix throughout in the context of healing ECM *in vivo* as it relates to scar-free tissue regeneration, a thorough review of Seifert has failed to reveal any disclosure of production of anything like an ECM_{ev}. The closest that has been found to any such disclosure in Seifert is found on page 17 which provides:

"For histological and immunohistochemical analysis, **harvested tissues were fixed in 10% NBF** at 4°C for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4°C and **processed for paraffin embedding**. Samples were cut at 5mm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4uC, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT."

NBF is Neutral Buffered Formalin, or formaldehyde, a cross-linking agent. If the Examiner is asserting that Seifert's histological section preparation steps of fixing and embedding a tissue specimen and the product produced anticipates a process used to prepare tissue product and the

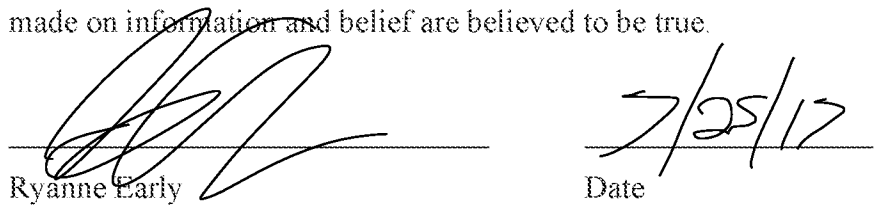
product produced which is suitable for implantation, then it is my opinion that such assertion is simply illogical. Accordingly, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of retaining structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

7. I am aware that claims 5, 20-22, 26 and 34-48 stand rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

It is my opinion that the combination proposed by the Examiner in rejecting the claims is not based on sound reasoning and is made only with hindsight, after study of the present application and claims. The Examiner has conceded that Asem does not teach the ECM wherein the amphibian is a Urodele or axolotl. To address this deficit in Asem, the Examiner combines the Asem teaching with that of Seifert. However, as noted above in response to the anticipation rejection based on Seifert, the only mention of ECM in Seifert is in the context of healing ECM *in vivo* as it relates to scar-free tissue regeneration. Further, as noted above, it is simply illogical to assert that Seifert's teaching of histological section preparation steps for fixing and embedding a tissue specimen and the product produced by such a method anticipates, teaches or suggests a process to prepare tissue product and the product produced which is suitable for implantation as a xenograft. Seifert neither teaches nor suggests the present process or product and, those skilled in the art aware of Asem would only have found in Seifert a study of the wound healing processes in the Axolotl.

8. For all of the foregoing reasons, it is my opinion that the references cited by the Examiner do not teach or suggest the claimed invention, that is, a sterile, isolated, decellularized Urodele extra cellular matrix (ECM) produced by a process comprising:
(A) obtaining a tissue sample from a Urodele, which tissue sample comprises extracellular matrix, cells and ground substance, and
(B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.



Ryanne Early

7/25/17

Date

RYANNE EARLY
ISE Professional Testing & Consulting Services, Inc.
5115 NW 67th Street,
Gainesville, Florida
(352) 474-0823
Early.Ryanne@gmail.com

Professional Profile

I am experienced in the discovery, development and strategic commercialization of innovative technologies in the fields of Orthopedic, Neurosurgical, Dental, and Surgical Specialties Medical Devices. I have participated as a scientist, development engineer, and quality assurance director in the development of multiple emerging technologies that are currently commercially available. As a previous Research and Development Manager of a mature medical device company and Quality Assurance Director of a start up medical device company, I have been responsible for technical and quality personnel teams that focused on supporting activities for innovative technology development and commercialization. Activities included strategic concept selection and evaluation; intellectual property and scientific/clinical literature assessment; State, FDA, and International registration and submission; design and creation of quality management and documentation management systems; and intelligent approaches to environmental control and safety. As a previous cofounder and current business owner, I have guided integrated approaches to achieve short and long term business and quality goals by implementing a "quality and business value by design" facility. I have designed and implemented product development process and operational systems that focused on compliance, efficiency, flexibility, patient and personnel safety, and clinical efficacy. Designs have exceeded requirements of tissue, device, and laboratory facilities. They also employ methodologies comparable to the pharmaceutical industry encompassing sustainable energy and waste efficiency strategies through utilization of state of the art information science technologies. I frequently provide services as a technical marketing expert to support clinician-industry relationship development. My previous roles involved closely working with marketing teams to function as the primary interface at the technical level, responsible for communicating and training on technical concepts and developing technical marketing strategies associated with distributing partners, end user clinicians, contract laboratories, and university research programs. This role requires frequent attendance and presentations at surgeon events on behalf of clients as an expert in the field of Regenerative Medicine and regulatory compliance.

Education

PhD Biomedical Engineering Unfinished	University of South Florida	Tampa, FL
MS Biomedical Engineering December 2004	California Poly State University	San Luis Obispo, CA
BS Engineering Science May 2003	University of Florida Biomechanics Minor	Gainesville, FL

Professional Experience

Chief Scientific Officer
Seed Biotech, Inc.
January 2012-Current

- Contract Manufacturing Company specializing in tissue based biomaterial & Advanced Process Capabilities
  - Designed and Implemented quality by design (QBD) manufacturing facility utilizing process analytical technology (PAT)
  - Developed advanced process technology for preparation of tissue based products in initial portfolio offering
  - Oversight for R&D, QA, and Operational Activities

Advisory Board Member
University of Florida
April 2011-Current

- Development of Curriculum, Solicitation & utilization of department funds
  - Graduate Program
  - Undergraduate Program

Founder & Consultant
ISE Professional Testing and Consulting Services, Inc.
May 2010-Current

- Technology Development
  - Novel Biomaterial Development (patent pending)
  - Advanced Process technology for allograft & xenograft tissue
  - Quality by Design Facility Design
  - Process Analytical Technology utilization for QA/QC of biological tissue processing

- Consulting Services

Post Market Support
Synthes, Inc.

Technical/Development/Manufacturing Svcs:
Musculoskeletal Transplant Foundation
McCaa Group
MiMedX Group, Inc.
Axogen, Inc.
TrueMotion Spine

Management Systems – QMS, Design Control:
Applied Biological Concepts, Inc.
Titan Spine, Inc
Surgical Biologics, Inc.
Exogenesis, Inc.
Xhale

Collaborative Research & Development
University of Florida & Florida State University

Consultant
MiMedx Group
Kennesaw, GA
February 2011 – December 2011

- Full-time field consultant clinical expert for industry leading advanced material technology company
  - Amniotic membrane and basic science expert
  - Clinical liaison for conscious use and benefit potential for material and product to consumers and users
  - Support mutual R&D and operations for existing and developing products
  - Design concept aligned with current technologies
  - Representative expert for the company

Quality Assurance Director & Co-Founder
Alpha Biosurgery, Inc.
Gainesville, Fl
May 2010 – January 2011

- Designed and Implemented a "Quality and Business Value by Design" Production Facility and Facility Support Infrastructure

  - Plan and Implement a Hybrid Quality Management and Document Management System (GTP, GLP, ISO 9001)
  - Design and Implement a Energy and Resource Efficient Fluid Management System
  - Design and Implement a Compliant Semi-Automatic End to End Item/Critical Environment Identification, Trace, Track, and Alert System with Remote Access capability from Supplier to End User. The three layer system infrastructure used FDA compliant RFID-EPC-Barcode, Online Cloud Interfaces, and wireless biosensors for item level control over production planning, production, processes, production

facility, material storage, and product inventory sustainment and has an integrated documentation database that maintains internal and external documentation.

Research and Development Department Manager, Surgical Specialties and Dental Division
RTI Biologics, Inc.
Alachua, Florida and Neunkirchen, Germany
February 2008 – January 2009

- Product Line Introduction: CopiOs Xenograft Membrane for Zimmer Dental for US Distribution (Launched Q3-2008). Responsible for the successful launch of novel processed Xenograft membrane for Zimmer Dental. Design elements included material characterization for indication specific needs of the product, Design Control Procedures including design transfer, design verification and design validation essential to the on time product launch.

- Engineering Manager, Tutoplast Processed Dermis Market Support Project. Managed the core team that designed analyses, collected, and evaluated data. I was the lead author for the marketing literature generated to support Tutoplast Processed Acellular Dermis and circulated in the Hernia, Breast Recon, Women's and Men's Health, ENT, and Dental Markets

- Engineering Manager and Technical Marketing Expert, Competitive Product Support Project for Puros Particulate. Developed a four phase competitive analysis project. I was invited and attended Zimmer Dental East and West Sales Training Seminars and Zimmer Dental 2009 National Sales Meeting to present Results and train sales representatives on the Positioning Strategy for the Puros Product Line.

- Research Project Manager, Development Project Manager, and Technical Lead, Novel Custom Engineered Allograft Dermis Connective Tissue Scaffold (Target Launch Hernia and Women's Health Markets Q3-2010). Technical and Project leader overseeing all project activities from design creation in the concept stage to verification and validation activities in the development stage. The scaffold customized for open homogenous porosity was strategically produced using existing material that is processed and unused engineered and shown to improve host tissue reaction and promote rapid cellular infiltration and revascularization while significantly improving returns on existing investments with minimal additional resource allocation. *

Research Scientist & Product Development Engineer: Spine, Surgical Specialties, Dental Division
Tutogen Medical, Inc.
Alachua, Florida and Neunkirchen, Germany
April 2007 – February 2008 (Merged with RTI)

- Lead Scientist/Engineer, Biomechanical and Biological Characterization of Existing Soft Tissue and Bone Product Portfolio. As a new R&D Department, my primary role was to characterize the existing product lines to provide technical data that would assist the competitive capabilities of the Company Product Portfolios and assist in Next Generation product lines. Responsible for setting up laboratories with essential capital equipment, and additionally the protocols and supportive still in use for emerging technologies evaluations.

- Lead Scientist/Engineer, Novel Rapid Rehydration Package. Designed and Developed Rapid Rehydration package for cortical and cancellous derived biological implants.*

- Lead Scientist/Engineer, Novel Precision Machined Press-Fit Intervetebral Disc Fusion Device Project. Developed and designed concept, coordinated design transfer to manufacturing facility, and wrote the G-Code for the CNC machine to optimize processing time and tissue utilization.*

Legal Associate
Michael A. Croak, Corporate Law Office
Central Florida
June 2005-February 2007

- Interim evaluation of career path in Intellectual Property Law. Prepared for LSAT and was accepted to multiple law schools.

Sr. Research Associate, Spine Biomechanics Lab
Neurosurgery Department, Tampa General Hospital
June 2003-June 2005

- Senior member of the team conducting an artificial disc replacement study. Responsibilities included preparing human spine specimens with strain gauges and extensometers, setting up testing equipment, and performing testing.

- Senior member of the team that designed a three point bending fixture with adjustable columns to perform beam testing on potential bone substitute materials.

- Senior member of a team conducting contract testing for Synthes including a three month *in vivo* study for on caprine animal spines with resorbable cervical fusion cages. Responsibilities included assisting in the surgical implant procedure, specimen preparation, testing setup, and running the biomechanical tests.

- Senior member of a team conducting contract testing for Medtronic on surgical optimal placement of interbody spacer. Responsibilities included assisting in the surgical implant procedure, specimen preparation, testing setup, and running the biomechanical tests

- Senior member of a team conducting contract testing for Pegasus Biologics on the Material Properties assessment and comparison of multiple cross linked equine pericardium configurations. Responsibilities included protocol development, testing rationale, test set up, and test execution.

- Participated in experimental surgical demonstrations, and additionally presented evaluations of emerging technologies to Neurosurgical Clinicians for consideration.

- Designed, fabricated, and installed an electronic intradiscular pressure measuring device.

- Designed an investigative study of the thoracolumbar and lumbar spinal junctions to reveal mechanical properties, specifically stiffness gradient across junctions that would assist in better medical device development. Responsibilities included preparing subsequent grant proposals for funding.

Core Skill Set Overview

Software

- Microsoft Word, Excel, PowerPoint,
- MTS software
- BlueHill Software (Instron)
- Minitab Statistical Software
- ImagePro Image Analysis Software
- Peak Motion Analysis System
- Autolev Motion Analysis Software
- SolidWorks
- Algor Finite Element Modeling (certified training)

Medical Device Project Manager Experience

- Concept Development and Testing
- Grant Proposal Preparation
- Design Control Procedures (FDA and ISO compliance for Design History File Compilation)
- Cross – Departmental coordination
- Packaging/s/sterilization
- Protocols, Risk and Regulatory Analysis
- Quality System Regulations (QSR)
- Intellectual Property Assessment
- Post Launch Product Support Including Sales Rep Training and Competitive Product Analysis
- Patent Bar to be taken December 11' (Patent Agent Status)

Professional Training

- Leadership Essentials Training

Clinical Experience

- 5 months clinical shadowing:
- 30 hours operating room experience (Neurosurgery and General Surgery)

Trained Methods of Evaluation

- Karl Fisher Moisture Analysis
- Histological Evaluation (qualitative and quantitative)
- Electron Dispersion System (EDS) for Elemental Characterization
- DSC Analysis for Cross linking and thermal stability assessments
- X-ray Diffraction
- Electron Scanning Microscope Prep and Analysis
- MicroIndentation
- Uniaxial and Biaxial Biomechanical Testing (including Visual Image Correlation)
- Extra Cellular Matrix Resorption Profile Determination using Collagenous Assay
- Precision CNC machining (G-Code)
- Advanced Statistical Evaluation and Trend Analysis
- DNA, RNA, Protein Assays

Professional Memberships

- Society of Women Engineers
- Society of Biomedical Engineers
- Orthopedic Research Society

Technical Marketing Engineer Experience

- Presenter Zimmer Dental 2008 East Sales Conference
- Presenter Zimmer Dental 2008 West Sales Conference
- Presenter Zimmer Dental 2009 National Sales Conference
- New Partner Product Platform Presenter for Entrigue Medical. 2008
- Presenter Davol 2010, Multiple Surgeon Events and Sales training event

Published Proceedings and Transactions (prior names Ryanne Hernandez)

"Viscoelastic Mode of Collagen Matrix: Mathematical Constitutive Model with Experimental Verification", M.Haile, R. Hernandez, P.Ifju, Society of Experimental Mechanics Conference, Orlando June 2008 (Presented)

"Viscoelastic Constitutive Model of Collagen Dermal Implants" M.A. Haile, R. Hernandez, J. Faleris, D. Greenspan, P.G Ifju, Society of Experimental Mechanics 2009 Annual Conference and Exposition, June 1-3, 09, Albuquerque, NM

"Determining Multiaxial Properties of Porous Viscoelastic Membrane using Bubble-inflation Test" M.A Haile, J. Faleris, R. Hernandez, D. Greenspan, P.G Ifju, Society of Experimental Mechanics 2009 Annual Conference and Exposition, June 1-3, 09, Albuquerque, NM

"Viscoelastic Characterization of Collagen Matrix in Time and Frequency Domains" M.A. Haile, R. Hernandez, D. Greenspan, P.G. Ifju , Society of Experimental Mechanics 2009 Annual Conference and Exposition, June 1-3, 09, Albuquerque, NM

"The Collagen Matrix of Human Dermis: Mechnical and Histological Characterization" R. Hernandez, J. Faleris, A. Poniatowki, D. Greenspan, Society of Biomaterials 2009 Annual Conference, San Antonia, Texas (Presented)

"Non-crosslinked Tutoplast® Bovine Pericardium and Human Dermis: an *in vivo* Comparison" R. Hernandez, J. Faleris. American Hernia Society Nation 2010 Annual Conference, Orlando, Florida. (Presented)

"*In vivo* Evaluation of an Autologous graft and two Acellular Human Dermal Matrices" J. Faleris, R. Hernandez. Society of Biomaterials 2010 Annual Conference, Seattle, Washington.

"Characterization of Puros® DBM Putty" S. Moore, J. Katz, R. Zhukauskas, R. Hernandez, R. Dodds, C. Lewis, S. Grover, N. Long, R. Cobb. Society of Biomaterials 2009 Annual Conference, San Antonia, Texas (Published)

"*In vivo* and *in vitro* histological evaluation of two commercially available acellular dermal matrices" J. Faleris, R. Hernandez, D. Wetzel, R. Dodds, D. Greenspan. Hernia Journal. October 2010. (Published)

"Biomechanics and Enzymatic Degradation of Two Acellular Dermal Matrices" Faleris JF, Michaelson JD, Hernandez RMC, Moore ST, Cobb R. Society of Biomaterials 2011 Annual Conference, Orlando, Fl (Submitted)

Additional Research Experience

Research Assistant 2003
Performed micro indentation on equine cortical bone to investigate the influence of treatment, anatomical region, and osteon type on indentation moduli and hardness.

Solid Biomechanics Research 2002
Research included investigation on the osteon arrangement around the foramen of an equine third metacarpal. The research investigated the trajectory of osteon cells around the foramen with pursuit to relate the trajectory to the mechanical behavior exhibited in the bone around the foramen.

Fluid Biomechanics Research 2001
Intent of the research was to assess the effects of an herbal supplement on patients suffering from osteoarthritis. Tasks included measuring the viscosity of diseased synovial fluid over a treatment period. The research introduced the concept of testing the viscosity of synovial fluid using a Brookesfeild and Microrheometer as a future diagnosis method for osteoarthritis.

*Proprietary information of RTIBiologics, Inc.

Electronic Acknowledgement Receipt

EFS ID:	29889116
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Eileen MacKenzie
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	25-JUL-2017
Filing Date:	08-JUL-2015
Time Stamp:	19:49:43
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Affidavit-traversing rejectns or objectns rule 132	S186070000US01-AFF-ERG.PDF	134885 08a40c7c1833202e07f2ae3db5655043e9797951	no	8

Warnings:

Information:					
2	Miscellaneous Incoming Letter	S186070000US01-EARLY_CV-ERG.PDF	167486 7e65716fcecfc086f907c85a60a9870b66e4d36b	no	6
Warnings:					
Information:					
			Total Files Size (in bytes):	302371	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	**140**
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	**140**

APPLICATION AS AMENDED – PART II

AMENDMENT **07/25/2017**

	(Column 1)		(Column 2)	(Column 3)		
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 59	Minus	** 48	= 11	X $40 =	440
Independent (37 CFR 1.16(h))	* 9	Minus	***11	= 0	X $210 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	**440**

AMENDMENT

	(Column 1)		(Column 2)	(Column 3)		
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
GLORIA J. TRAMMELL

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CONSIDERED: /R.A.D/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; **371 date: Jul. 8, 2015** : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

INVENTOR'S DECLARATION UNDER 37 C.F.R. 1.132
TO ACCOMPANY THE RESPONSE, UNDER 37 C.F.R. 111, TO NON-FINAL OFFICE ACTION, MAILED ON 01/25/2017

TO: THE COMMISSIONER OF PATENTS:
& EXAMINER DAVIS

1. I hereby declare that I am the same Ryanne Early named as a sole inventor in the subject pending patent application, and whose CV is attached to this Declaration.

2. I am aware that the Examiner in the subject application, in an Office Action dated 1/25/2017, has rejected all pending claims as being a product of nature, as being anticipated by cited art and as obvious in light of a combination of cited art. I am also aware of and have read the Amendments to the claims and arguments in support of patentability that are being filed concurrently with this Declaration.

3. It is my opinion that it is incorrect that the claims in the subject application are directed to a law of nature or to a natural product. *In vivo,* there is a structure that is referred to as "extracellular matrix", (ECM) which is not the same material, morphologically, physicochemically, biochemically, exhibiting different surface chemistry interaction potential and functionality as compared with that which exists *ex vivo,* albeit also referred to by the term "extracellular matrix" or "ECM". For clarity, adopting the terminology used in the attached

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Amendment, ECM as it exists *in vivo* is referred to as ECM_{iv}, while ECM as it exists *ex vivo* is referred to herein as ECM_{ev}.

ECM_{iv} performs the function of providing a structural support for cells - a scaffold of connective tissue to which is attached (via, e.g. integrins and/or other attachments) a wide range of molecules, cells, membranes, proteins and the like. The ECM_{ev} according to this invention has been denuded of intact cellular material. In addition, a wide array of chemical bonds are broken, cellular material is removed, and biochemical constituents changed, such that ECM_{ev} is, in fact, very different from ECM_{iv}.

FIG. 5 in the subject application provides a histological evaluation of H&E-stained, paired native and post-processed sections of axolotl dermal tissue. Clearly, even from just a gross morphological perspective, the ECM_{iv} and ECM_{ev} are quite different, if for no other reason than that the ECM_{ev} is essentially devoid of cells.

FIG. 6 presents ELISA data for DNA content in pre- and post-processed axolotl tissue, with comparison data from human amniotic membrane tissue. It is evident from this data alone that ECM_{ev} produced according to the method of this invention contains approximately 90+% less DNA than does native ECM. This demonstrates that intact cells and cellular debris has been removed, providing a porous structure that is now conductive and can support ingrowth, which is not the case for native ECM. Furthermore, the density, deformation, and absorption characteristics of ECM_{ev} differs significantly to that of native ECM_{iv}.

If ECM_{iv} were to be analogized with genomic DNA, it is noted that while genomic DNA *in vivo* can exist in excised forms, ECM tissue does not exist in the body in an excised form. That is, *in vivo*, it would be an oxymoron to refer to "decellularized extracellular matrix", as ECM is laid down by cells, at appropriate developmental stages, by the cells that form it and in which they themselves are integral. Accordingly, formation of ECM_{ev} from ECM_{iv} is analogous to the chemical and physical transformation required to produce a cDNA.

What is clear from the data presented in Figures 5 and 6 in the present patent disclosure is that ECM_{iv} is an intricate series of structures, layers, and conduits, tightly interlaced with cells, which

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contain DNA), cell components, proteins, and the like. ECM_{iv}, replete as it is with cells and structure accommodating those cells, is in no way the same as ECM_{ev} following the decellularization process taught in the subject patent disclosure. Per this disclosure, ECM as it exists *in vivo*, i.e. ECM_{iv}, is isolated, extraneous molecules, cells and tissue is removed, to produce Urodele connective tissue, (synonymous herein with ECM_{ev}), characterized by retention of structural and functional integrity coupled with reduced or eliminated antigenicity, due to removal of sufficient cellular components to achieve that characteristic. Nothing like this is disclosed, taught or suggested in Seifert.

While intact native tissue, as shown in Seifert, is a composite material consisting of cells (interstitial and basement membrane), Extra Cellular Matrix (fibrous protein, non-fibrous protein, and glycosaminoglycans), and ground substance, the biomaterial claimed in the present application has been found to exhibit beneficial bioinductive and bioconductive qualities, as compared with native tissue in which cells preoccupy adhesion sites, exhibit minimum binding affinity, and exhibit such antigenicity as to be unusable as a xenograft.

Transplanar discontinuity, which is evident only in excised vs. intact tissue, exhibits a fracture surface of ECM which is otherwise continuous in native tissue. This fracture surface has additional adhesion sites and permits for ingrowth into pores in the matrix vs. solely via facial surface in native tissue, and exhibits altered pore kinetics. This contributes to altered reaction rates ordinarily observed only in native tissue restricted to facial surface access, mass transport and diffusion. It also contributes to altered stability since the opportunity and rate of hydrolysis additionally increases.

Native soft connective tissue exhibits trans-planar continuity, existing in a state of tensintegrity, that is concurrent radial compression and tension. This leads to physical geometry indicative of any material under such stresses: deformation, reduced thickness, and tensioning of interwoven fibers. Because these features further impact on resultant porosity, pore kinetics, and masked or available adhesion sites, additional contributions to structural and function properties occur. However, once native tissue has been clarified or decellularized, the remaining biomaterial from decellurallized urodele tissue is formed into three-dimensional shapes as desired. It is optimized for binding pharmaceutical agents, for subsequent efficient secondary processing, including

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solubilzation and covalent tethering, and loading, which cannot be done with native tissue due to challenges caused by cellular material or other impurities.

The biomaterial comprised of optimized isolated and decellularized ECM according to this invention additionally is signifiacntly more thermally stable becfause of its lack free H_20, and because of the ECM exposure to divalent oxygen there is no way to effectively eliminate the generation of free radicals within the biomaterial. Absent these key feartures, there would exist limited ability to subject the biomaterial to ionizing radiation as is done in terminal sterilization, because high moiusture native tissue would in the same condition undergo signifaicnt alteration involving concurrently the disruption of native covalent bonds in and between ECM and cells. In addition, new unnatural covalent bonds in and between ECM and cellular materials would be formed because of the high variability and lack of normal distriburtion of properties and consituents within and between tissues of the same and different animals. There is no practical means to implement use of terminal sterilization in native tissue beause of the inconsistency of imparted unique atlerations which would result even when all controllable parameters are maintained with tight tolerances. The same exact challenges present for lyphoization based secondary processing where varying celular impurities impair the ability to prepare formed shapes of material using freeze drying.

4. I am aware that claim(s) 5, 20-22, 34-37, 45 and 46 are rejected under 35 USC 102al as being anticipated by Smith et a1. (1982).

It is my opinion that Smith teaches nothing whatever about ECM *per se,* i.e. neither ECM*iv* nor ECM*ev* is taught or suggested by Smith. A review of the Materials and Methods section of Smith, page 244, left hand column, corroborates this assertion. There it is disclosed that the *Ambystoma* is decapitated "and the skins and cartilages dissected from other tissues and placed on ice." Note, there is no decellularization of the tissue. Accordingly, this still only comprises ECM*iv*. The "skins were subsequently extracted in 0.4 ionic strength phosphate buffer, pH 7.6, and three changes of 0.5 M **acetic acid.** Each extract was clarified by centrifugation, and **the collagen in the supernatant was purified**".

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Thus, per Smith, an acid soluble fraction of collagen is isolated, purified and characterized. There is no isolation, purification, or characterization of ECM. Per the described process of Smith, any ECM present in the starting material would be found as a pellet in the centrifugation process, along with any other acid insoluble debris, such as cellular components and the like, with proteins such as collagens extracted into the supernatant. Accordingly, any ECM that existed in the starting material of Smith is actually **completely destroyed** and discarded by the extraction that Smith conducts. Reconsideration and withdrawal of this ground for rejection is respectfully requested.

5. I am further aware that Claims 5, 20 - 22, 34 - 39, 41, 44 — 47 are rejected under 35 U.S.C. 102al as being anticipated by Satoh et a1, (2012).

It is my opinion that Satoh does not teach an ECM_{ev} product that is the same or even similar to the presently claimed ECM_{ev}. At page 192, bottom of left hand column to the top of the right hand column, Satoh provides the following description of how an "acellular collagen sheet" was produced from Axolotl tissue:

"The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, **and then incubated in water with 0.01% Tween 20, PMSF (1.74 μg/ml), and glutaraldehyde** (20.18 μg/ml) for two days, resulting in an acellular ECM sheet. The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 3°C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed on the rerouted nerve."

Accordingly, Satoh does not disclose production of a material for use as a xenograft - the Satoh material is used on an Axolotl. Satoh also only disclose the production of a miniscule amount (due to the tiny amount of skin available from the Axolotl upper limb) of "an acellular ECM sheet". Furthermore, the Satoh material is obtained from an axolotl in the form of a cross-linked matrix (due to the glutaraldehyde) which would not retain the structural and functional integrity of the tissue sample. In addition, by cross-linking the material produced according to Satoh, both

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the cross linking itself and the fact that such cross linking would covalently bind any cellular debris still present (since Satoh teaches treatment of the entire native ECM with detergent to rupture cells and no separation of such material is taught prior to glutaraldehyde treatment), the Satoh material fails to meet the requirement in the present claims that sufficient cellular components are removed to reduce or eliminate antigenicity of the biomaterial as a xenograft. Per Satoh, this cannot be achieved due to the covalent cross-linking of cellular debris to the connective tissue matrix. Furthermore, glutaraldehyde treatment of proteins and cellular extracts generally increases antigenicity.

By contrast, the product according to the present invention is produced by a process comprising decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft. Accordingly, Satoh does not anticipate the present claims.

6. I am aware that claims 5, 22 and 47 are rejected under 35 USC, 102a1 as allegedly being anticipated by Seifert et al., (2012).

It is my opinion that while Seifert mentions ECM and extracellular matrix throughout in the context of healing ECM *in vivo* as it relates to scar-free tissue regeneration, a thorough review of Seifert has failed to reveal any disclosure of production of anything like an ECM_{ev}. The closest that has been found to any such disclosure in Seifert is found on page 17 which provides:

"For histological and immunohistochemical analysis, **harvested tissues were fixed in 10% NBF** at 4°C for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4°C and **processed for paraffin embedding**. Samples were cut at 5mm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4uC, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT."

NBF is Neutral Buffered Formalin, or formaldehyde, a cross-linking agent. If the Examiner is asserting that Seifert's histological section preparation steps of fixing and embedding a tissue specimen and the product produced anticipates a process used to prepare tissue product and the

product produced which is suitable for implantation, then it is my opinion that such assertion is simply illogical. Accordingly, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of retaining structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

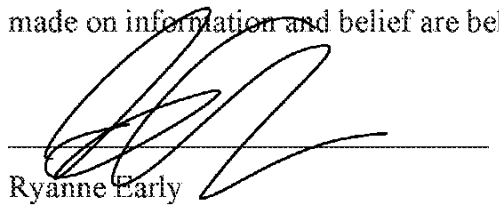
7. I am aware that claims 5, 20-22, 26 and 34-48 stand rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

It is my opinion that the combination proposed by the Examiner in rejecting the claims is not based on sound reasoning and is made only with hindsight, after study of the present application and claims. The Examiner has conceded that Asem does not teach the ECM wherein the amphibian is a Urodele or axolotl. To address this deficit in Asem, the Examiner combines the Asem teaching with that of Seifert. However, as noted above in response to the anticipation rejection based on Seifert, the only mention of ECM in Seifert is in the context of healing ECM *in vivo* as it relates to scar-free tissue regeneration. Further, as noted above, it is simply illogical to assert that Seifert's teaching of histological section preparation steps for fixing and embedding a tissue specimen and the product produced by such a method anticipates, teaches or suggests a process to prepare tissue product and the product produced which is suitable for implantation as a xenograft. Seifert neither teaches nor suggests the present process or product and, those skilled in the art aware of Asem would only have found in Seifert a study of the wound healing processes in the Axolotl.

8. For all of the foregoing reasons, it is my opinion that the references cited by the Examiner do not teach or suggest the claimed invention, that is, a sterile, isolated, decellularized Urodele extra cellular matrix (ECM) produced by a process comprising:
(A) obtaining a tissue sample from a Urodele, which tissue sample comprises extracellular matrix, cells and ground substance, and
(B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

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The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.



Ryanne Early

7/25/12

Date

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	Art Unit
	RUTH A DAVIS	1651

CPC - Searched*		
Symbol	Date	Examiner
A61K 35/65	10/11/2017	rad

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
east, pubmed, google patent	10/11/2017	rad
A61K 35/65	10/11/2017	rad

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01	3358

23628 7590 10/18/2017
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EXAMINER	
DAVIS, RUTH A	

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
10/18/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 14/759,895	Applicant(s) Early, Ryanne	
	Examiner RUTH A DAVIS	Art Unit 1651	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 7/25/17 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☑ Claim(s) 1-33,36-38 and 40-61 is/are pending in the application.

5a) Of the above claim(s) 1-4,6-19,23-25 and 27-33 is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☑ Claim(s) 5,20-22,26,36-38 and 40-61 is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____ .

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____ .

4) ☐ Other: _____ .

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Applicant's amendment, reply and affidavit filed July 25, 2017 have been received and entered into the case. Claims 34, 35 and 39 are canceled; claims 49 – 61 are added. All arguments and the affidavit have been fully considered.

Claim Objections

Claim objections are withdrawn due to amendment.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 42, 44 and 61 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

In claim 42, the phrase "a human agent" is not adequately defined by the claim language or specification.

In claim 44, "a sterile derived Urodele fraction derived from the isolated, decellularized Urodele ECM" lacks sufficient antecedent basis. The limitation is also broader than the claim on which it depends.

Claim 61 is rendered indefinite because it is unclear what elements are in the alternative as proper Markush language is not recited, thereby failing to clearly delineate the scope of the claim.

Claim Rejections - 35 USC § 101

Rejections under 35 U.S.C. 101 are withdrawn due to amendment and applicant's reply.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.

Claims 5, 20 - 22, 36 - 38, 41, 44 and 47are rejected under 35 U.S.C. 102a1 as being anticipated by Satoh et al. (2012).

Satoh teaches acellular collagen and ECM sheets (decellularized ECM) from axolotl (p.192). Although Satoh does not teach each of the claimed process steps by which the collagen is produced, these limitations are considered to product by process type limitations. It is noted that the patentability of a product does not depend on its method of production. If the claimed product is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference), the claim is unpatentable even though the reference product was made by a different process. When the prior art discloses a product which reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C 102 and/or 35 U.S.C 103 are proper. (MPEP 2113) Regarding the intended use of decellularizing the ECM to retain structure and functional integrity of the ECM, this intended use of the claimed composition does not patentably distinguish the composition, per se, since such undisclosed use is inherent in the reference composition. In order to be limiting, the intended use must create a structural difference between the claimed composition and the composition of the prior art. In the instant case, the intended use fails to create a structural difference, thus, the intended use is not limiting. Please note that when applicant claims a composition in terms of function, and the composition of the prior art appears to be the same, the Examiner may make rejections under both 35 U.S.C 102 and 103 (MPEP 2112). Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen.

Therefore, the reference anticipates the claimed subject matter.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 5, 20 – 22, 26, 36 – 38, 40 - 61 are rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

Asem teaches decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col.5 line 23-39), derived from amphibians (col.4 line 35-40). The ECM may in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col.6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col.4 line 30-35) or with cell culture comprising growth factors for culturing human (col.11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col.7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45).

Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl. However, at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as

well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration.

Thus, the claims are rendered obvious.

Response to Arguments

Applicant argues that Satoh does not teach the ECM retains its structure and function or has reduced antigenicity and that it is crosslinked with glutaraldehyde which is a different structure. Applicant further argues that Seifert does not anticipate, teach or suggest making a tissue graft but teaches away from using the ECM in healing; that the rejection is not sound and makes no sense; and that the examiner uses improper hindsight.

However, these arguments fail to persuade for the following reasons.

Regarding Satoh the reference teaches a decellularized ECM sheet from a Urodele as claimed. The reference teaches an acellular ECM, or decellularized, which would inherently have the claimed retention of structural and functional integrity as claimed. Even if the ECM is crosslinked with glutaraldehyde, it would still retain sufficient structure and function to permit and support use of the matrix as a substrate for growth of cells in vivo or in vitro, as defined by the instant specification on page 12, thus inherently meeting the claimed limitations. Absent clear evidence of a structural or functional difference between the claimed ECM and that of Satoh, the arguments are found unpersuasive.

Regarding Seifert, it is noted that the rejection is a combination of Asem in view of Seifert and not Seifert alone. Asem is relied upon to teach decellularized ECM comprising basement membranes derived from amphibians wherein the ECM may in the form of sheets that are adhered together (or laminated), powders (or particles) or reconstituted gels, made under alkaline conditions (col.6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col.4 line 30-35) or with cell culture comprising growth factors for culturing human (col.11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col.7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). Seifert is relied upon to evidence that it would have been obvious to use Urodele or axolotl as a functionally equivalent amphibian ECM with a reasonable expectation for successfully obtaining a functional decellularized ECM. Thus, as stated in the rejection above, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Applicant's arguments are not found persuasive.

In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. See *In re McLaughlin*, 443 F.2d 1392, 170 USPQ 209 (CCPA 1971).

For these reasons, the claims remain rejected.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH A DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on Monday - Friday (8am - 4pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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/RUTH A DAVIS/
Primary Examiner, Art Unit 1651

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Application	Document	Mailroom Date	Attorney Docket No.
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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Request for Continued Examination (RCE) Transmittal		
Address to: Mail Stop RCE Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Application Number	14/759,895-Conf. #3358
	Filing Date	July 8, 2015
	First Named Inventor	Ryanne Early
	Art Unit	1651
	Examiner Name	R. A. Davis
	Attorney Docket Number	S1860.70000US01

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C 371, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO on page 2.)

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).
   - a. [ ] Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
     - i. [ ] Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
     - ii. [ ] Other _____
   - b. [X] Enclosed
     - i. [X] Amendment/Reply
     - ii. [X] Affidavit(s)/ Declaration(s)
     - iii. [ ] Information Disclosure Statement (IDS)
     - iv. [ ] Other _____
2. Miscellaneous
   - a. [ ] Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   - b. [ ] Other _____
3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   - a. [ ] The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 23/2825 .
     - i. [X] RCE fee required under 37 CFR 1.17(e)
     - ii. [ ] Extension of time fee (37 CFR 1.136 and 1.17)
     - iii. [ ] Other _____
   - b. [ ] Check in the amount of $ _____ enclosed
   - c. [X] Payment by credit card ~~(Form PTO-2038 enclosed)~~

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SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED			
Signature	/Edward R. Gates/	Date	March 14, 2018
Name (Print/Type)	Edward R. Gates	Registration No.	31,616

~~***CERTIFICATE OF MAILING OR TRANSMISSION***~~			
~~I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.~~			
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Dated: March 14, 2018 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

6136254.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services,
	: Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian
	: Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; **371 date: Jul. 8, 2015**
	: Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

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Dated: March 14, 2018 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

RESPONSE, INCLUDING A PETITION AND FEE FOR A TWO MONTH EXTENSION OF TIME, TO ACCOMPANY A REQUEST FOR CONTINUED EXAMINATION ("RCE") OF THE SUBJECT APPLICATION, WITH AMENDMENTS TO CLAIMS AND ARGUMENTS PRESENTED TO ADDRESS AND OVERCOME THE REJECTION OF CLAIMS BY THE EXAMINER IN THE FINAL OFFICE ACTION, MAILED IN THE SUBJECT APPLICATION ON 10/18/2017

THE COMMISSIONER OF PATENTS & EXAMINER DAVIS:

The claims pending in this application are claims 5, 20-22, 26 and 36-38 and 40-61, while claims 1-4, 6-19, 23-25, and 27-33, stand withdrawn from consideration. Per the Final Office Action mailed in the subject application on 10/18/2017, all claims under consideration stand rejected under 35 U.S.C. §§ 102 and 103. It is gratefully acknowledged that the rejection, under 35 U.S.C. §101, asserting the claims were directed to subject matter that is non-eligible for patent protection, under the product of nature exception to patentability, has been withdrawn.

Amendments to the claims begin on page 2 of this paper, and remarks and arguments in support of the patentability of the claims, as herein amended, begin on page 10 herein below.

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IN THE CLAIMS:

Please replace all prior listings of claims in this application with the following listing of claims. A clean set of claims, as herein amended, is provided as an attachment at the conclusion of this paper.

1. (Withdrawn) A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. (Withdrawn) The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. (Withdrawn) The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. (Withdrawn) The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. (Currently amended) A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample, by a process comprising:

(i) retaining the structural and functional integrity of said tissue sample; and

(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial <u>making it suitable for use</u> as a xenograft.

6. (Withdrawn) An isolated, decellularized Urodele ECM.

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7. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. (Withdrawn) The isolated ECM of claim 6, wherein the ECM includes basement membrane.

9. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. (Withdrawn) The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a sheet.

12. (Withdrawn) The isolated ECM of claim 11, wherein the sheet includes perforations.

13. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. (Withdrawn) The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

16. (Withdrawn) A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized

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Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. (Currently amended) A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample, by a process which comprises:

(i) retaining the structural and functional integrity of said tissue sample; and

(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

(iii) sterilizing said tissue.

21. (Original) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Currently amended) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample, by a process which comprises:

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(i) retaining the structural and functional integrity of said tissue sample; and
(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and
(iii) sterilizing said tissue.

23. (Withdrawn) A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. (Withdrawn) The tissue culture system of claim 22, wherein the cells are human cells.

25. (Withdrawn) Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. (Original) A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. (Withdrawn) A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. (Withdrawn) A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. (Withdrawn) The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

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30. (Withdrawn) The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. (Withdrawn) The method of claim 30, further comprising reconstituting the powder as a gel.

32. (Withdrawn) The method of claim 29, further comprising sterilizing the decellularized ECM.

33. (Withdrawn) The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

Cancel claims 34-35.

36. (Previously presented) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Previously presented) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. (Previously presented) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

40. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (Currently amended) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial

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agent, a synthetic polymer, an adhesive, a drug or ~~a human~~ an agent found in a human, whether isolated, synthetically or recombinantly produced.

43. (Previously presented) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. (Previously presented) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Previously presented) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Currently amended) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel, a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (Previously presented) The tissue graft of claim 49, wherein the ECM is a powder.

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51. (Previously presented) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (Previously presented) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (Previously presented) The tissue graft of claim 49, wherein the ECM is a paste.

54. (Previously presented) The tissue graft of claim 49, wherein the ECM is a gel.

55. (Previously presented) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (Previously presented) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (Previously presented) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (Previously presented) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (Previously presented) The tissue graft of claim 57, wherein the agent is a growth factor.

60. (Previously presented) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

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61 (Currently amended) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

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REMARKS

This paper is being filed to accompany a Request for Continued Examination in response to the final Office Action, dated 10/18/2017 in the subject application and the attached Expert Declaration under 37 CFR 1.132 of Dr. Ashley Seifert.

Claims 1-4, 6-19, and 23-25 and 27-33 stand withdrawn from consideration as being drawn to non-elected subject matter. Rejoinder of method claims upon allowance of composition of matter claims is requested.

Claims 34, 35, and 39 have been previously cancelled.

Accordingly, the claims under consideration in this application are claims 5, 20-22, 26 and 36-38, and 40-61.

I. 35 USC §112:

Claims 42, 44 and 61 stand rejected under 35 U.S.C. §112(b) or pre-AIA 35 U.S.C. §112 second paragraph, as being indefinite.

Claim 42 is rejected based on the phrase "a human agent" which is asserted to not be adequately defined by the claim language or specification.

To address and overcome this ground for rejection of claim 42, the claim is herein amended to recite "an agent found in a human, whether isolated, synthetically or recombinantly produced". This text is supported by the specification at paragraph 0029 which discloses: "a human agent (i.e., an agent found in a human, isolated, synthetically or recombinantly produced)."

Reconsideration and withdrawal of this ground for rejection is respectfully requested.

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Claim 44 is rejected as the text "a sterile derived Urodele fraction derived from the isolated, decellularized Urodele ECM" is said to lack antecedant basis and for being broader than the claim from which it depends.

To address and overcome this ground for rejection of claim 44, claim 20 is herein amended to recite "or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM", thus both providing antecedent basis and ensuring that the additional limitation included in claim 44 narrows that claim as compared to claim 20 from which it depends.

Reconsideration and withdrawal of this ground for rejection is respectfully requested.

Claim 61 is rejected as indefinite due to an alleged lack of clarity as to what elements are in the alternative due to improper Markush language.

To address and overcome this ground for rejection of claim 61, claim 49 is herein amended to recite a sheet, to thereby provide more fully explicit antecedent basis for that form of ECM recited in claim 61. Furthermore, claim 61 is herein amended to correct the improper Markush language.

Reconsideration and withdrawal of this ground for rejection is respectfully requested.

II. 35 USC §101

The Examiner's withdrawal on the record of the rejection of claims under 35 USC §101 is gratefully acknowledged.

III. 35 USC §102:

It is gratefully acknowledged that the prior rejections of claims in this application as being anticipated by Smith alone or by Seifert alone, have been withdrawn on the record, by virtue of having not been reiterated in the outstanding Office Action.

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Claim(s) 5, 20-22, 36-38, 41, 44, and 47 stand rejected under 35 USC 102al as being anticipated by Satoh et a1. (2012).

It is gratefully acknowledged that the prior rejection of claims 34 and 35, as anticipated by Satoh, has been withdrawn on the record, by virtue of having not been reiterated in the outstanding Office Action.

It is alleged that "Satoh teaches acellular collagen and ECM sheets (decellularized ECM) from axolotl (p192). **Although Satoh does not teach each of the claimed process steps by which the collagen is produced, these limitations are considered to product by process type limitations**. It is noted that the patentability of a product does not depend on its method of production, **if the claimed product is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference**), the claim is unpatentable **even though the reference product was made by a different process.** When the prior art discloses a product which **reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C. 102 and/or 35 U.S.C. 103 are proper**, (MPEP 2113). Regarding the intended use of decellularizing the ECM to retain structure and functional integrity of the ECM, this intended use of the claimed composition does not patentably distinguish the composition, *per se*, since such undisclosed use is inherent in the reference composition. In order to be limiting, the intended use must create a structural difference between the claimed composition and the composition of the prior art. In the instant case, the intended use fails to create a structural difference, thus, the intended use is not limiting. Please note that when applicant claims a composition in terms of function, and the composition of the prior art **appears to be the same**, the Examiner may make rejections under both **35 U.S.C. 102 and103** (MPEP 2112). Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen. Therefore, the reference anticipates the claimed subject matter."

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In the present case, it is urged that **it is unreasonable** to assert that the prior art product "appears to be **identical with** or **slightly different than the claimed product-by-process" product**. The Examiner does not allege that the process utilized by Satoh is the same or even similar to Applicant's process.

The Examiner clearly appreciates that the claims in the present application are in fact product claims, albeit constructed as product-by-process claims. That is to say, the product claims pending in this application have been drafted to intentionally include process limitations which, by virtue of being included as affirmative limitations in the wording of the claim and the production of a product encompassed by the claim, inherently, necessarily, *per force*, and without fail, results in the production of a product which is demonstrably different than an allegedly known "prior art product" made by an unquestionably different process.

With this concordance in appreciation of the product-by-process nature of the claims under examination in this application, it is urged, respectfully, that where, as here, the differences in the process steps required for production, on the one hand, of a "prior art" product, and, on the other hand, a product claimed in the format of a product-by-process claim, are so significant as to self-evidently produce very different products, then it should revert to the Examiner to demonstrate why a *prima facie* basis exists to assert that the products are identical or that they are only slightly different from each other.

In the present case, the inventor, and now another Expert, who happens to also be a consultant of one of the licensees of the patent applicant), are stating in the record their expert opinion that, as a result of the operation of the processes at issue, (i.e. in the present case, the claimed process involves removal of cellular debris and no cross-linking in the production of the ECM product, whereas the Satoh "prior art" process involves no removal of cellular debris, cross-linking of the product itself, and cross-linking of cellular debris to the ECM product), **it cannot properly be said that the products are identical or that they are only slightly different than each other**.

The Examiner maintains this ground for rejection, notwithstanding arguments previously presented, allegedly, because:

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a. Regarding the Satoh reference, it is stated that "the reference teaches a decellularized ECM sheet from a Urodele as claimed. The reference teaches an acellular ECM, or decellularized, which would **inherently have the claimed retention of structural and functional integrity as claimed.** Even if the ECM is crosslinked with glutaraldehyde, **it would still retain sufficient structure and function to permit and support use of the matrix as a substrate for growth of cells in vivo or in vitro, as defined by the instant specification on page 12, thus inherently meeting the claimed limitations**. Absent clear evidence of a structural or functional difference between the claimed ECM and that of Satoh, the arguments are found unpersuasive."

In response, as previously argued on the record, a correct reading of Satoh reveals, (see page 192, bottom of left hand column to the top of the right-hand column), the following description of how an "acellular collagen sheet" was produced from Axolotl tissue:

"The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, **and then incubated in water with 0.01% Tween 20, PMSF (1.74 μg/ml), and glutaraldehyde** (20.18 μg/ml) for two days, resulting in an acellular ECM sheet. The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 3^0C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed on the rerouted nerve."

Accordingly, Satoh discloses only the production of a miniscule amount of ECM obtained from an axolotl (i.e. skin from the Axolotl upper limb), and the process disclosed according to Satoh is very different to the product defined according to current claims, and fails to meet the requirement in the present claims of a decellularizing process comprising:

"(i) retaining the structural and functional integrity of said tissue sample; and
(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft."

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Per the above-quoted process of Satoh, only a cross-linked matrix (due to the glutaraldehyde) was produced which, due to the cross linking of the product itself and cross linking of cellular debris to the product, would not retain structural and functional integrity of the tissue sample prior to decellularization and cross-linking. The material produced according to Satoh results in the production of a cross-linked product, thereby changing the chemical nature of the product, as compared to the present product as claimed, which is not cross-linked. It has furthermore previously been brought to the Examiner's attention that such cross-linking would not only change the physico-chemical properties of the decellularized ECM, but it would also covalently bind cellular debris which, per the Satoh disclosure, is not removed prior to the cross-linking step; i.e. Satoh teaches treatment of the entire native ECM with detergent to rupture cells, and, without removing that debris (i.e. no separation of such material is taught prior to glutaraldehyde treatment), glutaraldehyde treating the soup of cellular debris and ECM. Accordingly, Satoh fails to meet the requirement in the present claims that sufficient cellular components are removed to reduce or eliminate antigenicity of the biomaterial as a xenograft, since glutaraldehyde treatment of proteins generally increases antigenicity, and cross-linking cellular debris to the cross-linked ECM means that a huge number and diversity of cellular antigenic material is associated with the Satoh ECM. By contrast, the presently claimed product is not cross-linked and cellular debris is removed, rather than retained and cross-linked to the ECM.

The previously submitted Declaration of Ryanne Early, which should be entitled to due evidentiary consideration on the record, supports these arguments. However, notwithstanding the foregoing arguments and the Declaratory evidence already of record, as noted above, the Examiner, without evidentiary support, states that an acellular ECM, or decellularized ECM, produced according to Satoh, would "**inherently have the claimed retention of structural and functional integrity as claimed"** and that, "Even if the ECM is crosslinked with glutaraldehyde, **it would still retain sufficient structure and function to permit and support use of the matrix as a substrate for growth of cells in vivo or in vitro, as defined by the instant specification on page 12, thus inherently meeting the claimed limitations**."

While it might be the case that axolotl ECM produced according to Satoh may "**retain sufficient structure and function to permit and support use of the matrix as a substrate for growth of**

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cells in vivo or in vitro", this has not been demonstrated by the Examiner, nor by Satoh, nor has it been demonstrated that such a cross-linked matrix with attached cellular debris meets the current claim **requirements** to remove sufficient cellular components of the sample to "reduce or eliminate antigenicity of the biomaterial as a xenograft."

The Examiner appears to be completely ignoring the different physical, chemical and the antigenic properties of such a cross-linked matrix, as compared to the product claimed herein, which, as noted above, would also include a massive load of cellular debris included in the cross-linked ECM. Furthermore, independent of intended use, it is clearly the case that the ECM of the present invention, which is not cross-linked and from which cellular debris has been removed, is not the same as a cross-linked ECM which also contains cellular debris, some of which is trapped and some of which is likely cross-linked to the Satoh product.

Accordingly, the intended use, and method of producing the currently claimed product, does create structural differences between the claimed composition and the composition of the prior art.

No basis has been provided for the Examiner's assertion that the Satoh reference discloses a product that **inherently** has the claimed retention of structural and functional integrity, and that such a matrix **inherently** meets the claimed limitations. The concept of inherency is inapposite where, as here, a claimed product and a prior art product are clearly physicochemically and functionally different from each other. The position asserted by the Examiner is equivalent to stating that an orange with its peel and an orange without its peel are **inherently the same or only slightly different** from each other. That is a self-evidently preposterous assertion. It is unclear how the invocation of the concept of **inherency** trumps the clear, unambiguous, and uncontested differences (from e.g. the perspectives of the appearance, constituent component, functionality, weight, color, shelf-life, method of production, intended use, etc.) between a peeled and unpeeled orange, or, indeed, between the cellular debris retaining and cross-linked product of Satoh and the cellular debris depleted and non-cross-linked product of the present claims? The Examiner has failed to establish a sustainable *prima facie* showing that the Satoh product and the currently claimed product are the same or insignificantly different from each

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other, whether intended use is considered or not. Absent such a showing, novetly of the product claims presented herein should be acknowledged.

To further support the, in the opinion of the undersigned, self-evident novelty of the ECM of the present invention, as compared to the cross-linked and cellular debris cross-linked ECM of Satoh, a further Expert Declaration, executed by Dr. Ashley Seifert, a consultant of a licensee of rights in the subject application, is provided herewith to provide support to the assertion that significant structural and functional differences exist between the claimed ECM and the product produced by Satoh as a result of the different methods of production disclosed.

Reconsideration and withdrawal of this ground for rejection is respectfully requested.

IV. 35 USC §103:

Claims 5, 20-22, 26, 36-38, and 40-61, stand rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

It is stated that "Asem teaches decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40). The ECM may [be] in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45)."

It is again gratefully acknowledged that the Examiner concedes that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, it is asserted that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM

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and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious."

It has previously been argued that the combination proposed by the Examiner in rejecting the claims is not based on sound reasoning. It is believed that the combination proposed by the Examiner does not make sense and is made only with hindsight, after study of the present application, in order to reject the claims. The Seifert et al. reference, if anything, teaches away from the combination proposed by the Examiner, and this is confirmed by Dr. Ashley Seifert in the attached Expert Declaration in which he comments on his own reference, namely, the Seifert reference relied on by the Examiner.

The Examiner asserts that it would have been obvious to use "any known functionally equivalent ECM" of the Asem follicular ECM as a substitute for the Asem follicular ECM, in spite of there being no teaching in Asem (or in Seifert et al.) that there is any tissue (other than placental tissue) that is the "functional equivalent" to follicular ECM. By contrast, as noted in the attached Declaration of Dr. Seifert, Asem teaches, if anything, that follicular tissue, like placental tissue, is unique.

Follicular ECM is found in small quantities and is relatively difficult to obtain. Asem teaches that follicular tissue is collected from eggs, including eggs of reptiles. The ECM is harvested from such tissue. Asem asserts that follicular tissue is more easily obtained, however, than placental tissue. The Examiner has provided no basis to support the assertion that ECM tissue sources other than follicular tissue would be the functional equivalent of ECM from follicular tissue. One of ordinary skill in the art would not go to the trouble of collecting small quantities of follicular tissue from eggs if any source of ECM could be readily substituted for follicular ECM.

Seifert does not suggest anything to the contrary. In fact, Seifert teaches that one would want to

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avoid the presence of ECM in promoting scar-free healing, teaching away from the combination suggested by the Examiner. For these reasons, the prior art does not teach or suggest the claimed invention, that is, a sterile, isolated, decellularized Urodele extra cellular matrix (ECM) produced by a process comprising "(A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft."

While Seifert mentions ECM and extracellular matrix throughout, this is all in the context of *in vivo* modelling of ECM production as it relates to scar tissue formation. In fact, a thorough review of Seifert has failed to reveal any disclosure of production of anything like an ECM_{ev}. The closest that has been found to any such disclosure in Seifert is found on page 17 which provides:

"For histological and immunohistochemical analysis, **harvested tissues were fixed in 10% NBF** at 4uC for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4uC and **processed for paraffin embedding**. Samples were cut at 5mm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4uC, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT."

NBF is Neutral Buffered Formalin, or formaldehyde, a cross-linking agent. Accordingly, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of decellularization and reduced antigenicity.

The Examiner maintains this ground for rejection, notwithstanding arguments previously presented, because: "Regarding Seifert, it is noted that the rejection is a combination of Asem in view of Seifert not Seifert alone. Asem is relied upon to teach decellularized ECM comprising basement membranes derived from amphibians wherein the ECM may [be] in the form of sheets that are adhered together (or laminated), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human

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(co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). Seifert is relied upon to evidence that it would have been obvious to use Urodele or axolotl as a functionally equivalent amphibian ECM with a reasonable expectation for successfully obtaining a functional decellularized ECM. Thus, as stated in the rejection above, at the time the claims were filed, it would have been obvious to use of ordinary skill in the art to use ECM from axolotl in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefits in cellular regeneration. Applicant's arguments are not found persuasive."

In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, the Examiner states that "it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such reconstruction is proper. See *In re McLaughlin,* 443 F. 2d 1392, 170 USPQ 209 (CCPA 1971). "

In response to the maintained rejection for obviousness, notwithstanding the foregoing arguments presented on the record, Dr. Ashley Seifert, the principal author in the Seifert publication being cited by the Examiner in combination with the Asem reference, provides his executed Declaration stating his concurrence with the arguments presented herein and contesting the position adopted by the Examiner.

It is recognized, at this stage in the prosecution, that neither the Ashley reference nor the Asem reference is being cited alone. Dr. Seifert, in his attached Declaration, makes it clear that it is his opinion, for reasons that have sound basis in scientific, chemical, compositional and physical property analysis, that his own publication is being improperly cited in combination with the Asem reference to support a proposition that is not supported by the Seifert publication, when considered alone or in combination with the Asem reference.

Dr. Seifert's Declaration makes it plain that follicular ECM and its mode of preparation, per Asem, would result in production of a product that, based on its method of manufacture and

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source, is a *sui generis* product. Given that the Asem reference neither teaches nor suggests use of Urodele or axolotl follicular ECM, it is evident that the Examiner's motivation to search for Seifert would be based on impermissible hindsight reconstruction from knowledge gained by reviewing the present disclosure and claims, and not from reviewing the Asem reference. Had the Examiner been simply operating as one of ordinary skill in the art to which Asem is directed also become apprised of the Seifert reference, that ordinarily skilled artisan would only have learned from the Seifert reference something about the laying down of ECM during wound free healing of axolotl injuries, and a method, including formalin fixation, much as in Satoh, to produce a product that is clearly different from that which is disclosed and claimed in the present application. The same applies to the analytical product produced according to the Seifert reference. The Asem reference, (Abstract), states: "A composition comprising follicle basement membrane is described. The composition can be utilized as a cell culture substrate for proliferating cells in vitro." Thus, Asem's intended use is not as a xenograft. The Asem disclosure relating to source for basement membrane neither mentions nor calls out Urodeles, nor axolotls, providing only the extremely generic mention of species which might conceivably include Urodele or axolotl: "The extracellular matrix prepared in accordance with one embodiment of the present invention comprises basement membranes isolated from the follicle tissues of egg laying vertebrate species including fish, amphibians, reptiles and birds. One preferred source of follicle tissue is the membrana granulosa of avian and reptile species."

In light of the foregoing arguments, and the expert opinion of Dr. Ashley Seifert, it is urged that the Seifert reference fails to cure the deficits noted in the Asem reference with respect to providing an enabling teaching or suggestion of the product or the process of its production as disclosed and claimed herein.

Reconsideration and withdrawal of this ground for rejection is respectfully requested.

SUMMARY:

All grounds for objection or rejection of claims asserted in the outstanding Office Action have been addressed and overcome herein by Amendment of the claims and by the remarks, arguments, and evidence, including that provided by Dr. Ashley Seifert, in the attached

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Declaration. The objections/rejections under 35 USC 112 have been addressed by claim amendments. The rejection of claims under 35 USC 101 as being drawn to ineligible subject matter has been withdrawn. Likewise, it is urged, the rejection of claims under 35 USC 102 and 103 have been overcome by claim amendments and by the arguments and evidence provided herein and in the Declaration of Dr. Seifert.

It is respectfully urged that all claims pending in this application are in condition for allowance.

In the event a telephonic or in-person interview would assist in bringing prosecution in this application to a successful conclusion, the Examiner is invited to contact the undersigned to arrange such an interview.

Dated: March 14, 2018

Respectfully submitted,

By: /Edward R. Gates/

Edward R. Gates
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CLEAN LISTING OF ALL CLAIMS PENDING (ALBEIT WITHDRAWN BUT NOT CANCELED) IN THIS APPLICATION:

1. A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample, by a process comprising:

(i) retaining the structural and functional integrity of said tissue sample; and

(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

6. An isolated, decellularized Urodele ECM.

7. The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. The isolated ECM of claim 6, wherein the ECM includes basement membrane.

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9. The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. The isolated ECM of claim 6, wherein the ECM is a sheet.

12. The isolated ECM of claim 11, wherein the sheet includes perforations.

13. The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

16. A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

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20. A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample, by a process which comprises:

(i) retaining the structural and functional integrity of said tissue sample; and

(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

(iii) sterilizing said tissue.

21. The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample, by a process which comprises:

(i) retaining the structural and functional integrity of said tissue sample; and

(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

(iii) sterilizing said tissue.

23. A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. The tissue culture system of claim 22, wherein the cells are human cells.

25. Tissue culture medium conditioned with isolated Urodele decellularized ECM or a

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Urodele fraction derived from isolated, decellularized Urodele ECM.

26. A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. The method of claim 30, further comprising reconstituting the powder as a gel.

32. The method of claim 29, further comprising sterilizing the decellularized ECM.

33. The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

36. The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

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37. The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. The tissue graft of claim 5, wherein the Urodele is an Axolotl.

40. The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or an agent found in a human, whether isolated, synthetically or recombinantly produced.

43. The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

46. The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

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48. The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel, a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. The tissue graft of claim 49, wherein the ECM is a powder.

51. The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. The tissue graft of claim 49, wherein the ECM is a paste.

54. The tissue graft of claim 49, wherein the ECM is a gel.

55. The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. The tissue graft of claim 49, wherein the ECM is a mesh.

57. A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

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58. The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. The tissue graft of claim 57, wherein the agent is a growth factor.

60. The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to .an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, : Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian : Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; 371 date: Jul. 8, 2015 : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

EXPERT DECLARATION UNDER 37 C.F.R. 1.132
TO ACCOMPANY THE FILING OF A REQUEST FOR CONTINUED EXAMINATION IN THE SUBJECT APPLICATION

THE COMMISSIONER OF PATENTS & EXAMINER DAVIS:

1. I hereby declare that I am the same Ashley W. Seifert whose *Curriculum Vitae* is attached to this Declaration, and that I am the same Ashley W. Seifert named as first author in the 2012 publication "Skin Regeneration in Adult Axolotls: A Blueprint for Scar-Free Healing in Vertebrates", (citable as PLoS ONE 7(4): e32875. doi:10.1371/journal.pone.0032875), which has been cited on the record by the Patent Examiner to support rejection of the claims pending in the subject application as lacking novelty or, in combination with other references, as being unpatentable for obviousness. I hereby further note that I am retained as a consultant by a licensee of rights in the subject application.

2. I am aware that the Examiner, in an Office Action dated 10/18/2017, has rejected certain claims as lacking novelty, in light of Satoh et al. (2012). I am also aware of, have read, and I concur with the arguments and claim amendments that are being filed concurrently with this Declaration, in support of the novelty of the pending claims, notwithstanding citation of the Satoh reference. Furthermore, I have read and concur with the previously submitted Declaration of Dr. Ryanne Early, the inventor in the subject application, filed to support the patentability of the pending claims. The ECM_{iv} and ECM_{ev} nomenclature provided in that Declaration is adopted and

referred to herein, along with an additional term defined herein, namely "ECM_{evxx}", used to refer to a cross-linked ECM_{ev} product produced according to the Satoh reference, as discussed further herein below.

3. It is my opinion that the product disclosed and claimed in the subject application (referred to herein as ECM_{ev}) is a necessarily different product (physicochemically and biochemically, exhibiting different surface chemistry interaction potential and functionality) than the glutaraldehyde cross-linked product ("ECM_{evxx}") the production of which is disclosed in the Satoh reference.

4. FIG. 5 in the subject application provides a histological evaluation of H&E-stained, paired native and post-processed sections of axolotl skin tissue. Clearly, even from just a gross morphological perspective, ECM_{iv} and ECM_{ev} are quite different, if for no other reason than that, in the ECM_{ev}, the cellular constituents present in ECM_{iv} have been removed. I understand that the Patent Examiner has recognized this and many other previously identified distinctions between ECM_{iv} and ECM_{ev}, by withdrawing a previously asserted rejection based on 35 U.S.C. section 101; thereby recognizing that the ECM_{ev} claimed in this patent application is not the same material or product as ECM_{iv} albeit retaining the natural architecture thereof.

5. In executing this Declaration, I am providing my opinion, as an expert in the field of tissue regeneration, (and the underlying and related fields, to the extent practically necessary, of, for example, biochemistry, tissue histological examination, microscopy, chemistry, and biology), that the Satoh product, created after removal of a sample of ECM_{iv} from its *in vivo* environment and subsequently processed, including via glutaraldehyde fixation, results in a product, ECM_{evxx}, which differs in a number of significant ways, as further attested to below, from ECM_{ev} and from ECM_{iv}, just as ECM_{ev} differs in a number of significant ways from ECM_{iv}. (please see the Table below in section 8).

6. As noted in Paragraph 4 herein, without more, simply the irrefutable basis that the Satoh product has been produced by a process that includes fixation, thereby producing ECM_{evxx}, it is my opinion that it is excluded from the claimed subject matter, it is different from the claimed

subject matter, and Satoh does not teach or suggest the subject matter taught and claimed in the subject application. What is claimed excludes, by its express terms, a tissue sample that has undergone fixation by treatment with glutaraldehyde[1], formaldehyde, (aka formalin), or combinations thereof, in that the present invention as claimed requires:

"obtaining a tissue sample from a urodele [i.e. axolotl, thus far, as in Satoh, the same ECM_{iv} starting material) where the tissue sample retains the **extracellular matrix** (ECM) which has been decellularized **by a process which preserves the structural and functional integrity of the ECM that it had in the ECM_{iv} tissue sample**." This is certainly not the case for the Satoh material if for no other reason than it has undergone glutaraldehyde fixation. While cells may have been disrupted in the course of production of Satoh's ECM_{evxx} product, in that product, pursuant to its method of production, disrupted cellular components can be retained in the cross-linked ECM as a result of glutaraldehyde treatment of the ECM in the presence of cellular debris.

7. I further opine that the intended use of the acellular material produced according to Satoh is revealed on page 194 of Satoh, right hand column, which provides commentary on Figure 6 in Satoh. Essentially, what Satoh reports is that the glutaraldehyde fixed, acellular collagen sheet of ECM_{evxx}, when placed in an environment otherwise conducive for accessory limb regeneration to be observed, (nerve rerouting or deviation + skin wounding), **the presence of the ECM_{evxx} prevents blastema formation and limb regeneration in the axolotl species from which the tissue was obtained.** Thus, quite the contrary to finding a demonstration in Satoh that an ECM_{ev} product could be produced with sufficiently depleted cellular components to make the product suitable for use as a xenograft, Satoh actually shows that in the axolotl, the ECM_{evxx} product prevents regeneration. This in no way can stand as a showing that the ECM_{evxx} is the same as

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[1] See, for example, https://en.wikipedia.org/wiki/Glutaraldehyde, which provides: "Glutaraldehyde is used in biochemistry applications as an amine-reactive homobifunctional **crosslinker** and **fixative** prior to SDS-PAGE, staining, or electron microscopy. **It kills cells quickly** by **crosslinking their proteins** and is usually employed alone or mixed **with formaldehyde** as the first of two fixative processes to stabilize specimens such as bacteria, plant material, and human cells. A second fixative procedure uses osmium tetroxide to crosslink and stabilize cell and organelle membrane lipids. Fixation is usually followed by dehydration of the tissue in ethanol or acetone, followed by embedding in an epoxy resin or acrylic resin. Another application for treatment of proteins with glutaraldehyde is the inactivation of bacterial toxins to create toxoid vaccines, e.g., the pertussis (whooping cough) toxoid component in the Boostrix Tdap vaccine produced by GlaxoSmithKline. In a related application, glutaraldehyde is sometimes employed in the tanning of leather and in embalming."

ECM_{ev} according to the present invention, nor would it inspire one skilled in the art to explore use of axolotl tissue for the purpose of making a tissue product suitable for use as a xenograft.

8. It is my opinion that the following table makes it clear that ECM_{evxx} is at least as dissimilar (non-identical) to either ECM_{iv} or ECM_{ev}, as ECM_{ev} is dissimilar (non-identical) to ECM_{iv}, particularly when considering the known presence or absence of particular cellular components, and the known function/intended-use of the product:

	ECM_{iv}	**ECM_{ev}** (Present invention)	**ECM_{evxx}** (Satoh)
	Present (P)/Absent (A) (function or intended use)	Present (P)/Absent (A) (function or intended use)	Present (P)/Absent (A) (function or intended use)
Intact Cells	P, (natural architecture with cells present)	A, (natural architecture retained due to absence of cross-linking; cells and cellular debris removed)	A, (non-natural architecture due to cross-linking; cells destroyed but cellular debris present due to cross-linking)
DNA	P (contained within intact cells)	A (i.e. while the natural architecture is retained, cellular material has been removed, as evidenced by the reduced A260/A280 of ECM_{ev} as compared to ECM_{iv})	A (presumably DNase treatment has removed DNA; no mention is made of RNase and RNA or any other cellular components or debris retained by cross-linking)
Cross-linking	A	A	P
Function/intended-use of product	*In Vivo* ECM_{iv} is a primarily collagenous matrix which provides structural support and is laid down by and between cells as they grow adjacent each other.	ECM_{ev} per the present invention is ECM*iv* which, while maintaining the natural architecture of ECM*iv* (by avoiding the use of cross-linking agents which lock the naturally variable and flexible morphology of ECM_{iv} into cross-link constrained morphologies and configurations), has been debrided of cellular material to reduce antigenicity to make the material suitable for use as a xenograft.	ECM_{evxx}, per Satoh is ECM*iv* which has been treated by fixation, with cross-linking agents which lock the naturally variable and flexible morphology of ECM_{iv} into cross-link constrained morphologies and configurations, and which has been treated in such a way that although intact cells have been destroyed, it is evident from Satoh itself that nuclei have been retained and require DNase treatment to remove that material. That is, the intended use of the Satoh product was to interfere with natural interactions at the injury site to inhibit regeneration, which is contrary to the intended use of the claimed product.

9. As previously stated in the Declaration of inventor Ryanne Early, what is clear from the data presented in Figure 5 in the present patent disclosure is that ECM_{iv} is an intricate series of

structures, layers, and conduits, tightly interlaced with cells, (which contain DNA), cell components, proteins, and the like. ECM_{iv}, replete as it is with cells and structure accommodating those cells, is not **"the same"** as ECM_{ev} following decellularization and removal of cellular debris, according to the process of production taught and claimed in the subject patent disclosure. Per this disclosure, ECM as it exists *in vivo*, i.e. ECM_{iv}, is isolated, nucleic acids are removed as a marker of removal of cells, and cellular debris is removed, to produce Urodele connective tissue, (also referred to herein as ECM_{ev}), characterized by retention of structural and functional integrity coupled with reduced or eliminated antigenicity, due to removal of sufficient cellular components to achieve that characteristic, making it suitable for use as a xenograft. Nothing like this is disclosed, taught or suggested in the Satoh reference, which is cross-linked to itself and to cellular debris. That is, even if it were contended that Satoh "attempts" to create something similar to what is disclosed and claimed in the present application, the Satoh processing is different in that fixation is used. Thus, to the extent that Satoh discloses or suggests production of an acellular graft, Satoh does so through different process steps, which results in production of a different product, for a different purpose.

10. It is my opinion that Satoh does not teach an ECM_{ev} product that is the same or even similar to the presently claimed ECM_{ev}. This opinion is based, at least in part, on the disclosure in the Satoh reference, at page 192, bottom of left hand column to the top of the right-hand column, where Satoh provides the following description of how an "acellular collagen sheet" was produced from axolotl tissue:

"The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, **and then incubated in water with 0.01% Tween 20[2], PMSF (1.74 μg/ml), and glutaraldehyde** (20.18 μg/ml) for two days, resulting in an acellular [3]ECM sheet. **The remaining**

[2] While this Declaration and the prior Declaration of Dr. Ryanne Early, and the remarks and arguments that have been made on the record have focused primarily on Satoh's teaching of the use of glutaraldehyde in the course of preparing the ECM_{evxx} product, it should not be ignored that Satoh also teaches the use of Tween 20, a detergent, and PMSF, a protease inhibitor

[3] Perhaps "acellular" in the sense of no remaining intact cells, but certainly not free from cellular debris - as is clear from the very next sentence, addressing the "remaining nuclei" which are removed by extensive DNase treatment. There would be no need to remove this material at this stage in the Satoh process had those materials not been trapped by the fixation process employed by Satoh. Inherently, in the process for manufacture of the ECM_{ev} product of the present invention, which makes no mention of use of

nuclei were removed by incubating the sheet in DNase solution for 1 h at 3°C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS **and placed on the rerouted nerve."**

In my opinion, the Satoh process, and the product made by that process, may properly be defined as:

Step a:

Isolate skin from the upper limb of an axolotl and incubate the skin sample in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours; the EDTA being present to sequester metal ions; intact cells present in the ECM_{iv} sample may or may not rupture during this step; if the cells rupture, the cellular components, such as nucleic acids, (see below) are not removed.

Step b:

Incubate the material from Step a in water with 0.01% Tween 20[4], PMSF (1.74 μg/ml), and glutaraldehyde (20.18 μg/ml) for two days, resulting in an acellular [5] ECM sheet.

Step c:

The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 3°C (Promega).[6]

Step d:

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[4] While this Declaration and the prior Declaration of Dr. Ryanne Early, and the remarks and arguments that have been made on the record have focused primarily on Satoh's teaching of the use of glutaraldehyde in the course of preparing the ECM_{evxx} product, it should not be ignored that Satoh also teaches the use of Tween 20, a detergent, and PMSF, a protease inhibitor, the use of either or both of which will have additional effects on the ECM_{evxx} of Satoh as compared to the ECM_{ev} of the present invention.

[5] Perhaps "acellular" in the sense of no remaining intact cells, but certainly not free from cellular debris - as is clear from the very next sentence, addressing the "remaining nuclei" which are removed by extensive DNase treatment. There would be no need to remove this material at this stage in the Satoh process had the nuclei, and presumably other cellular components, including other organelles, proteins, etc. not been trapped by the fixation process employed by Satoh. Inherently, in the process for manufacture of the ECM_{ev} product of the present invention, the effect of these other agents we be additive to create additional differences between the Satoh product and the product of the subject invention, beyond the effect of just the cross-linking that occurs in step (b) due to the presence of glutaraldehyde.

[6] As noted above, there would be no need to remove these nuclei had they not been trapped by the cross-linking step (b).

The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed *in vivo* on top of the rerouted nerve. While FITC staining is not necessarily specifically excluded in the process of manufacture of the product of the subject invention, it is certainly not required in that process. It is included in the Satoh process so that the inclusion of green fluorescent dye could be used to demonstrate placement of the ECM_{evxx} and that the ECM_{evxx} prevented interaction of the deviated nerve and the epidermis when introduced into the Satoh model. The product produced by this process is a cross-linked tissue in which the cells have been disrupted, but the debris from the disrupted cells is not necessarily removed, and which is FITC labeled.

11. Satoh also only discloses the production of a miniscule amount (due to the tiny amount of skin available from the axolotl upper limb) of "an acellular ECM sheet". Furthermore, the Satoh material is obtained from an axolotl in the form of a cross-linked matrix (due to the glutaraldehyde) which would fix and retain, in a given confirmation, the structure of ECM, while altering functional integrity of the tissue by changing access to active sites on the ECM itself. Glutaraldehyde cross-linking results in production of a much stiffer ECM material with non-native chemistry, physical properties, antigenic/immunogenic profile, and resorption/remodeling profile. In addition, by cross-linking the material produced according to Satoh, both the cross linking itself and the fact that such cross linking would covalently bind any cellular debris still present (since Satoh teaches treatment of the entire native ECM with detergent to rupture cells without teaching any separation of such material prior to glutaraldehyde treatment), the Satoh material fails to meet the requirement in the present claims that sufficient cellular components are removed to reduce or eliminate antigenicity of the biomaterial as a xenograft. Per Satoh, this cannot be achieved due to the covalent cross-linking of cellular debris to the connective tissue matrix.

12. By contrast, the product according to the present invention is produced by a process comprising decellularizing of the sample to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to render it suitable for use as a xenograft while retaining structural and functional integrity of the natural, cellular, tissue. Accordingly, the product and process of producing the product disclosed in Satoh is neither the same as or even

similar to the product and process of producing the product pursuant to the claims pending in the subject application.

13. I am aware that Claims 5, 20-22, 26, 36-38, and 40-61, stand rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012); that Asem is being cited as providing a teaching of decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40); that the Asem ECM is said to be in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64); that the ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). I further understand that the Examiner has conceded that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, that notwithstanding, I further understand that the Examiner is asserting that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious."

14. It is my opinion, as an expert in this field, and as principal author in the Seifert publication which the Examiner is citing, in combination with the Asem reference, that the combination proposed by the Examiner in rejecting the claims is not defensible. For the additional reasons that follow, it is my opinion that the Seifert et al. reference, if anything, teaches away from the combination with Asem proposed by the Examiner, in particular because the Seifert reference (page 15, bottom left column under the heading "ECM Production and New Tissue Formation"),

reports that "...the slow deposition of new extracellular matrix (ECM) and its unique molecular composition suggests newly synthesized axolotl ECM may antagonize fibrosis and promote regeneration (Figure 11)". Furthermore, (see page 16, left column, bottom before next heading), where it is stated in Seifert that: "These data suggest that the composition of ECM produced during a regenerative response may have an anti-fibrotic effect that helps to recapitulate embryonic development and promote a regenerative response". Thus, in fact, one of ordinary skill in the art encountering Seifert, while being apprised of Asem might understand that it would be worth trying to see if components of regenerating ECM, rather than intact, uninjured ECM, as disclosed and claimed in the subject application, may be useful as a tissue graft. Success in achieving this result is not disclosed in Seifert alone, in Asem alone, or in any combination of the two references. Furthermore, in my opinion, there is no basis provided in the Seifert publication to support the proposition that the Seifert reference provides "evidence that it would have been obvious to use Urodele or axolotl as a functionally equivalent amphibian ECM with a reasonable expectation for successfully obtaining a functional decellularized ECM." The Examiner has not provided any citation to any aspect of the Seifert publication which would support this allegation.

15. The Examiner asserts that it would have been obvious to use "any known functionally equivalent ECM" of the Asem follicular ECM as a substitute for the Asem follicular ECM. There is no teaching in Asem (or in Seifert et al.) that there is any tissue (other than placental tissue) that is the "functional equivalent" to follicular ECM. Asem teaches, if anything, that follicular tissue, like placental tissue, is unique, and the processes for making the product in Asem, as in Seifert, as in Satoh, as indeed in the present application, is *sui generis*. The Asem reference is further distinguished as being directed to basement membrane, which is not ECM *per se,* but only one constituent of ECM. Furthermore, Asem sources their basement membrane from a unique source, the ovarian follicle, which is essentially a thin basement membrane surrounding an oocyte (egg). In contrast, the ECMev according to the present invention is derived from mature skin, which is completely different in structure, form and function than the Asem basement membrane material, and was selected not for the basement membrane, but for the dermal tissue in combination with the basement membrane.

16. Follicular ECM is found in small quantities and is relatively difficult to obtain. Asem teaches that follicular basement membrane tissue is collected from eggs, including eggs of reptiles. The basement membrane ECM is harvested from such tissue. Asem asserts that follicular tissue is more easily obtained, however, than placental tissue. There is no basis to suggest that ECM tissue sources other than follicular tissue would be the functional equivalent of basement membrane ECM from follicular tissue. One of ordinary skill in the art would not go to the trouble of collecting small quantities of follicular tissue from eggs if any source of ECM could be readily substituted for follicular ECM. Furthermore, even assuming Asem were read as leading one skilled in the art to consider obtaining follicular ECM from a Urodele or even from an axolotl - Seifert does not teach production of the ECM produced according to the present invention; neither does Satoh; neither does Asem; neither do any or all of them combined do so. Thus, ECM produced according to Asem, even if made from Urodele or even axolotl basement membrane follicular tissue, would be a different product than if that same follicular tissue were obtained from Urodele or axolotl follicular tissue and treated according to the process of the present claims. This is made plain from Figure 1 of the Seifert reference, annotated to show tissues which are included in ECM_{ev} following decellularization to remove cells and debris form the dermis and basement membrane. The basement membrane is a tissue structure itself, but also a component of any epithelial tissue. The basement membrane is a component of the epidermis, intestine, etc., whereas ECM is constituted by a large class of proteins which primarily exist outside cells. For instance, the human MATRISOME, comprises over 200 proteins (http://web.mit.edu/hyneslab/matrisome/). Accordingly, in my opinion, citing the Asem reference, which is focused on use of basement membrane (comprised of just a few types of proteins) and asserting that such a structure is the same as or anticipates a product comprising ECMev, is equivalent to asserting that a simple protein formed from just a few types of amino acids (e.g. albumin), because it is formed from amino acids, is equivalent to, or is the same as or anticipates all the proteins on earth, which is not the case even though all proteins share some basic amino acids.

17. Seifert does not suggest anything to the contrary. In fact, Seifert teaches that one would want to avoid the presence of native ECM in promoting scar-free healing, teaching away from the combination suggested by the Examiner. For these reasons, it is my opinion that the cited

Satoh, Asem, and Seifert references, considered alone or in combination, do not teach or suggest a native (uninjured), sterile, isolated, decellularized Urodele extracellular matrix (ECM) produced by a process comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial thereby making it suitable for use as a xenograft."

18. While Seifert mentions ECM and extracellular matrix throughout, this is all in the context of *in vivo* ECM production as it relates to regeneration or scar tissue formation. In fact, a thorough review of Seifert has failed to reveal any disclosure of production of anything like an ECM_{ev} acquired as native ECM. The closest that has been found to any such disclosure in Seifert is found on page 17 which provides:

"For histological and immunohistochemical analysis, **harvested tissues were fixed in 10% NBF** at 4°C for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4°C and **processed for paraffin embedding**. Samples were cut at 5μm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4°C, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT."

NBF is Neutral Buffered Formalin, or formaldehyde, a cross-linking agent. Accordingly, much like the *ex vivo* tissue produced by Satoh, the Seifert material, whether considered alone or in combination with the Asem publication, fails to teach or suggest a decellularized ECM product with reduced antigenicity.

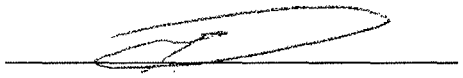
19. It is my opinion that it cannot validly be stated that Seifert's histological section preparation steps, carried out to produce tissue samples adapted for analysis of the scar free tissue regeneration process that occurs *in vivo* in the axolotl, which includes formalin fixing axolotl tissue specimens at different stages of *in vivo* wound healing, is adequate to teach or suggests a process used to prepare a tissue product, or the tissue product produced thereby, which is not formalin fixed, is decellularized, and thereby made suitable for implantation into another species than that from which it was derived.

20. In my opinion, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of retaining structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft.

21. It is my opinion that the combination proposed by the Examiner in rejecting the claims is not based on sound reasoning and is made only with hindsight, after study of the present application and claims. The Examiner has conceded that Asem does not teach the ECM wherein the amphibian is a Urodele or axolotl. To address this deficit in Asem, the Examiner combines the Asem teaching with that of Seifert. However, the only mention of ECM in Seifert is in the context of ECM produced *in vivo* in response to injury and its role in scar-free tissue regeneration within that species. Further, as noted above, it is simply illogical to assert that Seifert's teaching of histological section preparation steps for fixing and embedding a tissue specimen and the product produced by such a method anticipates, teaches or suggests a process to prepare a tissue product and the product produced which is suitable for implantation as a xenograft. Seifert neither teaches nor suggests the present process or product. Those skilled in the art aware of Asem's teaching, had they turned to or become aware of the Seifert reference, at most, may have thereby considered the possibility of acquiring ECM produced in response to injury, rather than, as in the subject application, using native, uninjured ECM_{ev}, which does not contain the same components as the ECM produced during healing *in vivo* as defined in detail in the Seifert publication.

22. For all of the foregoing reasons, it is my opinion that the references cited by the Examiner do not teach or suggest the claimed invention, alone or in combination, that is a decellularized Urodele extracellular matrix (ECM) produced by a process comprising:
(A) obtaining a tissue sample from a Urodele, which tissue sample comprises extracellular matrix, cells and ground substance, and
(B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft.

The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.



02 - 15 - 2018

Ashley W. Seifert, Ph.D. Date

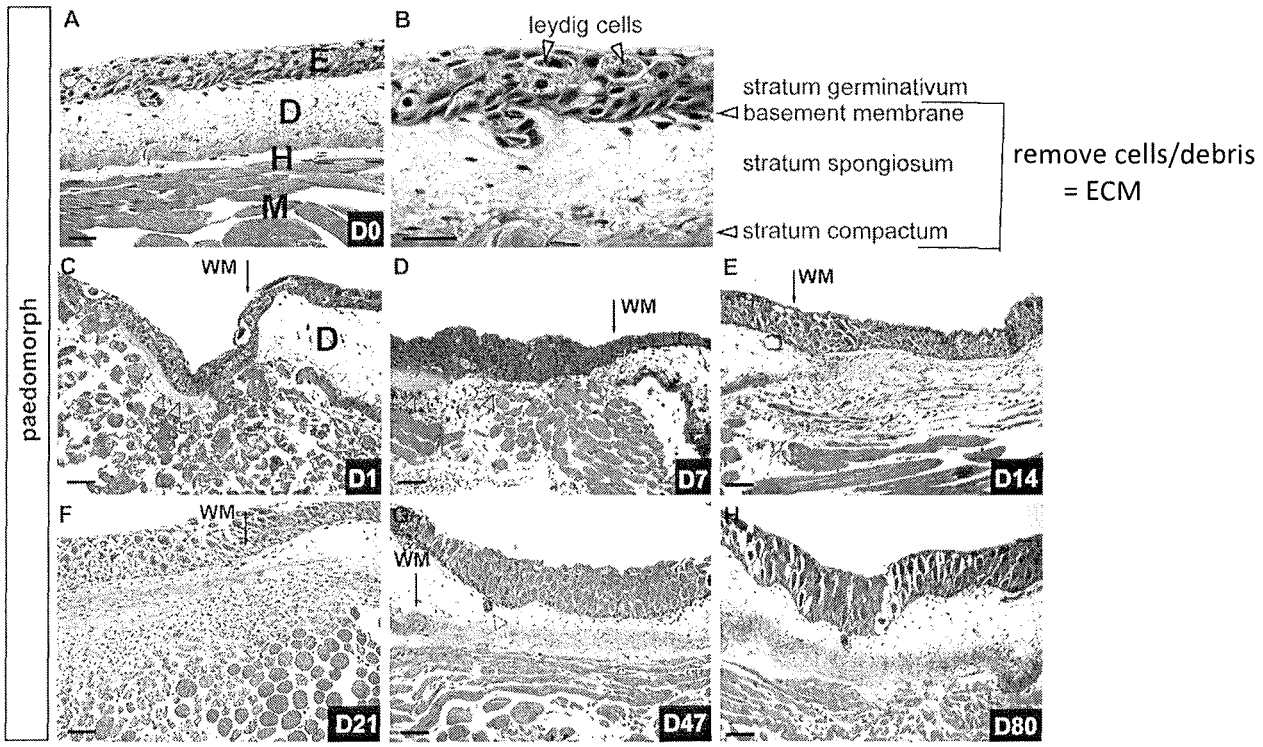


Figure 1. Scar-free healing of full thickness excisional wounds in adult axolotls. A) Masson's trichrome staining of uninjured dorsal flank skin in axolotls showing epidermis (E), dermis (D), hypodermis (H) and underlying muscle (M). B) Magnified image of epidermis and dermis. The epidermis is pseudo-stratified and contains epithelial cells and leydig cells (yellow arrows) while the dermis is divided into the stratum spongiosum (containing glands and dermal fibroblasts) atop the densely compacted ECM of the stratum compactum. C-H) Scar-free healing over 80 day period following full thickness excisional wounding. C) One day post injury (dpi) the wound bed is completely re-epithelialized. Some blood plasma has accumulated beneath the neoepidermis (green arrows) (wound margin = WM). D) Seven dpi there is little evidence of a fibrin clot between the epidermis and underlying muscle and no new ECM has been deposited. Green arrows depict residual blood plasma. E) Fourteen dpi fibroblasts are visible beneath the epidermis where new ECM is deposited (blue staining) and muscle cells are fragmenting into individual myoblasts. F) Twenty-one dpi a thick band of transitional matrix is visible beneath the epidermis. Collagen is visible within the regenerating muscle. G) Forty-seven dpi the underlying muscle has completely regenerated and is devoid of collagen. Skin glands (yellow arrow) have regenerated and descended from the epidermis. The dermal stratum spongiosum has reformed and the stratum compactum is coalescing. H) Eighty dpi all skin layers have regenerated. Scale bars = 100 mm.

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2010-Present	Visiting Professor, University of Nairobi, Department of Veterinary Anatomy and Physiology

EDUCATION

2009-2013	Post-doctoral Fellow, **University of Florida**, Department of Biology Mentor: Malcolm Maden, Ph.D.
2004-2009	**University of Florida,** Department of Zoology Ph.D., Developmental Biology, Advisor: Martin J. Cohn, Ph.D.
2008	SDB Short Course: *Frontiers in Developmental Biology: Concepts, Techniques and Model Organisms.*
2001-2004	**University of Florida,** Department of Zoology M.Sc., Physiological Ecology, Advisor: Lauren J. Chapman, Ph.D.
1994-1998	**Bowdoin College** A.B., Biology; minor in Philosophy, concentration in Photography

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Kimani, J.M., Kiama, S.G., Towett, P.K., and **Seifert, A.W**. Morphological adaptations to subterranean life: comparative anatomy of the skin from the African mole rat (*Tachyoryctes ibeanus)* (in revision *Journal of Morphology*)

Cyr, J. L., Gawriluk, T. R., Rada, B., Wendy T. Watford, W. T., **Seifert, A. W**., and Ezenwa, V. O. (*in revision*). Comparison of neutrophils from a regeneration-competent and incompetent mammal reveals subtle differences in cell quantity and function. *Frontiers in Immunology: Comparative Immunology*.

Cook, A. B., Shaw, D. and **Seifert, A. W**. (*in revision*). Normal skin development in salamanders reveals two routes to skin regeneration. *Development*

Book Chapters, Edited Volumes, Technical Reports, Other

Seifert, A.W. (2017). Comparative Regeneration. *Developmental Cell 43*. p. 373.

Coots, P., and **Seifert, A.W**. (2015). Thyroxine induced metamorphosis in the axolotl (*Ambystoma mexicanum*). In, *Salamanders in Regeneration Research* in Methods in Molecular Biology, eds. Kumar, A. and Simon, A. Springer. USA. pp. 141-145.

Ankerson, T.T., Fernandez, S., M., **Seifert, A.W**., and Velez, M.V. (2003). The gopher tortoise and upland habitat protection in Florida: Legal and policy considerations. In, Report on protected species and habitat protection, Ankerson, T.T., Fernandez, S.M., and Seifert, A.W. (eds). Report available through UF Levin College of Law library.

GRANTS, FELLOWSHIPS AND INDUSTRY FUNDING

2017-2022	R01 NIH - NIAMS, *Macrophage phenotype orchestrates tissue regeneration in mammals* (**PI**) –
2016-2019	Fundacao de Ciencia y Tecnologia (Portugal) (**International Collaborator**) - *Regeneration in African Spiny Mice*
2015-2018	NextGen Biologics Inc. (**PI**) - *Developing an amphibian sourced surgical mesh*
2015	Igniting Research Collaboration, University of Kentucky (**Co-PI**) - Sphingolipids and delayed wound healing in diabetes.
2015	National Geographic Grants in Aid of Research (NGS) (**PI**) - *Innate immunity and regeneration in East African rodents*
2014-2018	NSF, Division of Integrated Organismal Systems (IOS) and Office of International Science and Engineering (ISE). (**PI**) – *Immune tradeoffs during tissue regeneration in mammals*
2012-2013	SEED Biotechnologies Corporation. (**PI**) – funding for technician and research into scar-free healing in axolotls
2012 (spring)	SEED Biotechnologies Corporation. (**PI**) – for continued study exploring skin regeneration in salamanders towards developing regenerative therapies in humans
2009-2013	NIH NIDDK Postdoctoral Fellowship in Regenerative Medicine NIH T32DK074367
2009-2013	Singer Biology Fund Award. (**Co-PI**) – Effects of body size, age and metamorphosis on regenerative ability in *Ambystoma mexicanum* (axolotl).
2008	Department of Zoology Research Grant.– Genetic control of external genital development in mammals.
2008	HHMI GATOR Research Award. – Fibroblast growth factor expression during external genital development.
2001-2004	Grinter Fellowship. – Graduate fellowship awarded by University of Florida to cover summer research expenses
2003	National Science Foundation GRF (Honorable mention)
2002	Raney Fund, ASIH. – Metabolism across ontogeny in the African lungfish, *Protopterus aethiopicus.*
2002	Sigma Xi Grants in Aid of Research.) -- Energetics of the African lungfish, *Protopterus aethiopicus*

AWARDS

2011	Southeast Society for Developmental Biology Meeting -- Best Talk – awarded travel to SDB main meeting
2008	Society for Developmental Biology -- Best Poster, SDB Short Course (Runner-Up)

TEACHING

2017	MDBIL Summer Course: Comparative Regenerative Biology (Instructor)
2017 - ongoing	BIO 429/629: Developmental Biology (every spring)
2016	BIO 199: Stemcats Research Experience
2015	BIO 425: Animal Development and Regeneration
2015 - ongoing	UKC 300/A&S 300 BioArt: Creativity, Experimentation and Methodology in Art and Science (every other spring)
2014	BIO 197 StemCats (Faculty Participant)
2014 - ongoing	BIO 770 Topics in regeneration biology (every Fall)
2014	BIO 770 Principles of Regeneration
2014	BIO 580 Stem cells, tissue engineering and regenerative medicine (Co-Instructor)

2012	Seminar in Regeneration Biology (instructor)
2011	Integrative Principles (guest lecturer)
2011-2013	Biology for Non-majors (guest lecturer)
2010-2011	Evolution of Regenerative Mechanisms (co-instructor)
2008-2010	Reproductive Biology (guest lecturer)
2009	Seminar in The Cell Cycle and Development (instructor)
2007-2008	Howard Hughes Mentor
2004-2005	Developmental Biology (teaching assistant)
2004-2005	Genes, Embryos and Evolution (teaching assistant)
2001-2004	Functional Vertebrate Anatomy (laboratory instructor)

MENTORING

Postdoctoral Scholars

Tom Gawriluk (2014-present). Department of Biology, University of Kentucky (Mentor)
Jennifer Simkin (2015-present). Department of Biology, University of Kentucky (Mentor)

Current

Graduate Students (Advisor)

Sandeep Saxena (2016-present). Ph.D student. Department of Biology, University of Kentucky
Sruthi Purushothaman (2014-present). Ph.D student. Department of Biology, University of Kentucky
Shishir Biswas (2014-present). Ph.D student. Department of Biology, University of Kentucky
John Kimani (2014-present). Ph.D student. Department of Veterinary Anatomy and Physiology, University of Nairobi (**Co-Advisor**)
Luc Dunoyer (2013-present). Ph.D. student. Department of Biology, University of Kentucky (**Co-Advisor**)

Graduate Students (Committee Member)

Kristyn Van Der Meulen (2017-present). Ph.D. student. Department of Biology, University of Kentucky
Alex Chen (2015-present). Ph.D. student. Department of Biomedical Engineering, University of Kentucky
Nicholas Kohrs (2015-present). M.Sc student. Department of Biomedical Engineering, University of Kentucky
Chanung Wang (2014-present). Ph.D. student. Department of Biology, University of Kentucky

Completed

John Kimani (2010-2013). M.Sc. Department of Veterinary Anatomy and Physiology, University of Nairobi. *Wound healing and regeneration in the naked mole rat, Heterocephalus glaber.* (**Co-Advisor**).
Tom Gawriluk (2013-2014). Ph.D. Candidate. Department of Biology, University of Kentucky. *Targeted knockout of Beclin-1 reveals an essential function in ovary and testis.* (**Committee Member**).
Adam Cook (2013-2015). M.Sc Candidate. Department of Biology, University of Kentucky. *Beryllium nitrate supports fibroblast migration as an essential component of skin and limb regeneration in axolotls.* (**Advisor**)
Peggy Coots (2013-2015). MFA Candidate, Department of Fine Arts, University of Kentucky. (**Committee Member**).
Stephanie Bryant (2014-2016). Ph.D. Candidate. Department of Biology, University of Kentucky (**Committee Member**).

Undergraduate Students

Current

Corin Hacker (2015-present). Cartilage regeneration in African spiny mice.

Completed (*indicates publication with student)

Madison Ginn (2017). Genetic basis of salamander limb development.
Aaron Hoback (2016-2017). Immunity and regeneration in African spiny mice.
Cassandra Wooley (2016-2017). Requirement for macrophages during regeneration
Malik Guidry (2014-2016). Molecular basis of pattern formation. NSF REU student
Bailey Gensheimer (2015-2016). Effect if stress induced glucocorticoids on tissue regeneration.
Juliana Lavey (2014-2016). Molecular basis of pattern formation.
*Douglas Shaw (2013-2016). Skin microbiome axolotls. (BIO 395 student)
Zara Ashraf (2013-2015). Blastema formation during spiny mouse ear hole regeneration. (BIO 395 student)
Gavin Hall (2013-2014). Wnt/ß-catenin signaling during skin development and regeneration in axolotls.
Oliver Olivarez (2012-2013). Role of ECM during skin regeneration in salamanders.
Kirabet Quintana-Ramos (2012, summer). MMP function during skin regeneration in metamorphic axolotls.
*Daima Avila (2012-2013). Ear hole regeneration in mammals.
Adam Cook (2012-2013). Evolution of regenerative mechanisms in Urodeles. (Graduate student in Biology, UK).
Natalie Nelson (2011-2012). Skin development in *Ambystoma mexicanum*.
Joshua Hochberger (2010-2012). Identifying developmental mechanisms regulating wound healing and regeneration in Anurans.
Conner Burnside (2010-present). Developmental biology of the integument in *Ambystoma mexicanum*
Sean Connelly (2010-2012). The effects of functional traits on regenerative ability in *Ambystoma mexicanum*
McKayla Spencer (2010-2011). The effects of functional traits on regenerative ability in *Ambystoma mexicanum*
Jenny Fernandez (2008-2009). OPT imaging of the murine external genitalia.
Liz Dantzler (2008-2009). Fgf signaling during sexual differentiation of the external genitalia. (*publication in prep, see above*).
Rickesh Patel. (2007-2009). Howard Hughes Mentoring Program. Development and cellular origin of the mammalian vagina.
*Marissa Gredler (2007-2008). Functional analysis of *FgfR2* in the genital tubercle.
Stacey Gray. (2007). Howard Hughes Mentoring Program. *Foxa1* and *Foxa2* regulation of perineum formation.
Christina Varvarikos. (2006-2007). Howard Hughes Mentoring Program. Fgf signaling during genital development.
Virginia Hoglund (2005). Histological analysis of the developing mouse genital tubercle.
Eric Rubin (2005-2007). Androgen signaling during mammalian urethragenesis.
Sarah Tanaka (2003). Analysis of Q_{10} measures in an air-breathing fish, *Protopterus aeithiopicus*
Michael Wasserman (2002). Aestivation in the lungfish, *Protopterus aeithiopicus*.

ORAL PRESENTATIONS

2018 Invited Speaker: *TBD,* The Molecular & Cellular Basis of Regeneration and Tissue Repair, Malta
2018 Invited Speaker: *TBD,* Frontiers in Veterinary Medicine Speaker Series, University of Calgary, Calgary

2018 Invited Speaker: Spiny mice and complex tissue regeneration in mammals, Smith College, Northampton, MA

2017 Invited Speaker: *African spiny mice as an emerging model of epimorphic regeneration in mammals*, CDB Symposium: Mavericks in Developmental Biology, RIKEN Center for Developmental Biology, Kobe, Japan

2017 Invited Speaker: *African spiny mice, tissue regeneration and the injury microenvironment*, Cornell University, Department of Molecular Medicine, Seminar Series, Ithaca, NY

2017 Invited Speaker: *Unraveling the mechanistic basis for regeneration in spiny mice*, SDB 75th Annual Meeting, Organogenesis and Regeneration Session, Minneapolis, MN

2017 Invited Speaker: *Using spiny mice to reveal cellular regulators of tissue regeneration*, University of Southern California, Department of Stem Cell Biology and Regenerative Medicine, Distinguished Speaker Seminar Series, Los Angeles, CA.

2017 Invited Speaker: *TBD*, Vanderbilt University Medical Center (VUMC), Pathology, Microbiology and Immunology (PMI) Seminar Series, Nashville, TN (cancelled)

2016 Invited Speaker: *Immune cell dynamics during epimorphic regeneration in spiny mice*, Medical University of South Carolina (MUSC), Department of Regenerative Medicine and Cell Biology, Charleston SC.

2016 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Stem Cell Society Singapore (SCSS), Symposium 2016: Modeling Cell Fate and Development, Biopolis, Singapore (did not attend)

2016 Invited Speaker: *Immune cell dynamics during epimorphic regeneration in spiny mice.* Max Plank Institute for Molecular Biomedicine, Muenster Germany.

2016 Invited Speaker. *Blastema formation and musculoskeletal regeneration in mammals.* Weldon School of Biomedical Engineering, Sept. 28th 2016.

2016 Invited Speaker: *Animal regeneration and development*, FASEB Educational Briefing, Capital Hill, Washington, D.C.

2016 Invited Speaker: *Epimorphic regeneration in African spiny mice.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA

2016 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Pasteur Institute, Department of Developmental and Stem Cell Biology, Paris, France

2015 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Wakeman Institute, Rutgers University, Departmental Seminar Series, Piscataway, NJ

2015 Invited Speaker: *Integrating art and biology: in the classroom, the laboratory and beyond.* George Church Laboratory, Department of Genetics, Harvard University, Cambridge, MA

2015 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Harvard Department of Stem Cell and Regenerative Medicine, Cambridge, MA

2015 Invited Speaker: *Comparative biology of vertebrate regeneration*, MDI Biological Laboratory, Symposium: Comparative Biology of Tissue Repair, Regeneration and Aging, Salisbury Cove, ME

2015 Invited Speaker: *Regeneration: From Axolotls to Acomys*, Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

2015 Invited Speaker: *Comparative biology of vertebrate regeneration*, Department of Biomedical Engineering, University of Kentucky, Lexington, KY

2015 Invited Speaker: *Complex tissue regeneration in African spiny mice*, Department of Craniofacial and Mesenchymal Biology, UCSF, San Francisco, CA

2014 Invited Speaker: *New insights into mammalian skin regeneration*
Department of Cell and Molecular Biology, Tulane University, New Orleans, LA

2014 Invited Speaker: *Understanding limitations on vertebrate organ regeneration.*
CDB Symposium, RIKEN: Regeneration of Organs: Programming and Self-Organization, Kobe,

Japan

2012 Invited Speaker: *Weak skin, spiny hairs and regeneration in African spiny mice (Acomys).* Department of Biology, Texas Tech University.

2012 Invited Speaker: *Skin shedding and tissue regeneration in African spiny mice (Acomys).* Keystone Meeting; Mechanisms of Whole Organ Regeneration, Breckenridge, CO.

2011 Invited Speaker: *Perfect Skin Regeneration: New Models for Scar-Free Healing.* Department of Biomedical Sciences, Cornell University.

2011 Selected Speaker: *Skin regeneration in adult axolotls: A blueprint for scar-free healing in vertebrates.* Southeast Society for Developmental Biology, University of Florida.

2010 Invited Speaker: *Development of the anogenital system in mammals.* University of Nairobi, Department of Veterinary Anatomy and Physiology.

2007 Selected Speaker: *Origin of the penile urethra and the proximodistal fate of the urethral epithelium.* Society for Developmental Biology, Cancun Mexico.

2004 Selected Speaker: *Respiratory allocation and the standard rate of metabolism in the African lungfish, Protopterus aethiopicus.* Society for Experimental Biology main meeting, Edinburgh, Scotland.

2003 Invited Speaker: *The gopher tortoise and upland habitat protection in Florida: Legal and policy considerations.* Protected Species and Upland Habitat Workshop, UF Levin College of Law, Gainesville, Florida.

UNIV. OF KENTUCKY PRESENTATIONS

2017 Regenerative Biology at UK, A&S College Donor's Dinner, University of Kentucky

2017 SCoBIRC Works in Progress Seminar Series, University of Kentucky

2014 Research in Animal Regeneration: STEMCATS Chat, University of Kentucky

2013 Models in Regeneration Biology, Dean's Circle, University of Kentucky

POSTER PRESENTATIONS

2017 Purushothaman, S. and **Seifert, A.W.** *Understanding urodele limb development in the context of tetrapod limb evolution.* 14th International Conference on Limb Development and Regeneration, July 23rd, 2017, Edinburgh, Scotland

2016 Biswas, S., and **Seifert, A.W**. *Mammalian blastema formation in the African spiny mouse.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2016 Purushothaman, S. and **Seifert, A.W.** *Understanding urodele limb regeneration in the context of tetrapod limb development.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2016 Gawriluk, T. R., Simkin, J., Thompson, K.L., Biswas, S., Kiama, S.G., Ezenwa V.O., and **Seifert, A.W**. Understanding the immune response to injury in a regenerating mammal (*Acomys spp.*). SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2016 Simkin, J.S., Gensel J.C., and **Seifert A.W.** *Inflammation: setting the stage for tissue regeneration.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2015 Simkin, J.S., Gawriluk T., Biswas S., Gensel J.C., and **Seifert A.W.** *Macrophage phenotype reveals fundamental differences between regeneration and scarring.* MDI Biological Laboratory, Symposium: Comparative Biology of Tissue Repair, Regeneration and Aging, Salisbury Cove, ME

2015 Cook, A.B., and **Seifert, A.W.** *Beryllium nitrate reveals fibroblast migration as an essential*

component of skin and limb regeneration in axolotls. MDI Biological Laboratory, Symposium: Comparative Biology of Tissue Repair, Regeneration and Aging, Salisbury Cove, ME

2010 **Seifert, A.W.,** Monaghan, J., and Maden, M. Skin regeneration in the Mexican axolotl *(Ambystoma mexicanum)*. Florida Genetics, Gainesville, Florida.

2010 **Seifert, A.W.,** Monaghan, J., and Maden, M. Skin regeneration in the Mexican axolotl *(Ambystoma mexicanum)*. EMBO Meeting; Molecular and cellular basis of tissue repair and regeneration, Sesimbra, Portugal.

2010 Monaghan, J., **Seifert, A.W.**, and Maden, M. Nerve-dependent gene expression in the epidermis of regenerating salamander limbs. EMBO Meeting; Molecular and cellular basis of tissue repair and regeneration, Sesimbra, Portugal.

2009 **Seifert, A.W.**, Ormerod, B.K., and Cohn, M.J. *Sonic hedgehog* regulates cell cycle kinetics during morphogenesis. Society for Developmental Biology 68th Annual Meeting, San Francisco, California.

2009 **Seifert, A.W.**, Yamaguchi, T., and Cohn, M.J. Functional and phylogenetic role of *Fgf8* during external genital development. Society for Developmental Biology 68th Annual Meeting, San Francisco, California.

2008 **Seifert, A.W.**, Ormerod, B.K., and Cohn, M.J. *Sonic hedgehog* regulates cell cycle kinetics during morphogenesis. Latin American Society for Developmental Biology, Buenos Aires, Argentina.

2007 **Seifert, A.W.**, Harfe, B, and Cohn, M.J. Multiphasic role of *Shh* during external genital development and cloacal septation. Gordon Research Conference; Developmental Biology, New London, New Hampshire.

2007 **Seifert, A.W.**, Harfe, B, and Cohn, M.J. Multiphasic role of *Shh* during external genital development and cloacal septation. Society for Developmental Biology 66th Annual Meeting, Cancun, Mexico.

2006 **Seifert, A.W.**, Harfe, B, and Cohn, M.J. Origin of the penile urethra and the proximodistal fate of the urethral epithelium. Society for Developmental Biology 65th Annual Meeting, Ann Arbor, Michigan.

CONSULTING POSITIONS

2015-present	Consultant, NeXtGen Biologics, Inc.
2011-present	Consultant, ISE Professional Testing and Consulting Services, Inc.
2010-2014	Scientific Advisory Board, Regenerative Medicine International

PROFESSIONAL AFFILIATIONS

Society for Developmental Biology

SERVICE

2017-ongoing	Univ. of Kentucky, International Partnerships and Research Committee
2017	Univ. of Kentucky Faculty Search Committee (Stem Cells and Reg. Biol.)
2016-ongoing	Univ. of Kentucky, Dept. of Biology Executive Committee
2016-ongoing	Society for Developmental Biology (SDB) Public Affairs Committee
2016	FASEB Educational Briefing to Congressional Members and Staff
2016	Co-Organizer, SDB 75th Annual Meeting Satellite Symposium on Regeneration
2014-2015	National Science Foundation (NSF) Reviewer
2015-ongoing	Univ. of Kentucky, Dept. of Biology, Seminar Committee
2013-2015	Univ. of Kentucky Graduate Affairs Committee (GAC)
2015	Univ. of Kentucky Faculty Search Committee (Int. Physiologist Faculty Hire)

2013	Univ. of Kentucky Faculty Search Committee (Dev. Biol. Faculty Hire)
2011-2013	Univ. of Florida Postdoctoral Affairs Advisory Committee
2012-2013	Postdoctoral Mentoring Program
2005	Dept. of Biology, Faculty Search Committee (Evolutionary Functional Morphology)
2004	Graduate Curriculum Committee
2007-2008	Vice President, Zoology Graduate Student Association

Granting Agencies – Ad Hoc Reviewer
National Geographic Society, Department of Defense (ARO), National Science Foundation (NSF), Swiss National Science Foundation (SNF)

Academic Journals – Ad Hoc Reviewer
Science Reports, Developmental Cell, PLosOne, Journal of Morphology, EvoDevo, Journal of Sexual Medicine, Faculty of 1000, JoVE, Regeneration, JEZ Part B: Molecular and Developmental Evolution, Development, Journal of Anatomy, Developmental Dynamics, BMC Biology, BMC Developmental Biology, Neurogenesis, Biology of Reproduction

Electronic Patent Application Fee Transmittal

Application Number:	14759895
Filing Date:	08-Jul-2015
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Filer:	Edward R. Gates/Nicole Forgrave
Attorney Docket Number:	S1860.70000US01

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
RCE- 1st Request	2801	1	650	650
		Total in USD ($)		**950**

Electronic Acknowledgement Receipt

EFS ID:	32051114
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Nicole Forgrave
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	14-MAR-2018
Filing Date:	08-JUL-2015
Time Stamp:	14:51:32
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$950
RAM confirmation Number	031518INTEFSW14550700
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	S186070000US01-TRN-ERG.pdf	25081 fe64b565513cd01fb3e3bba952548cea0a65db4f	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	S186070000US01-FEE-ERG.pdf	31353 2ad31cc294ee0489c473b1b9678894bc72f5a6cb	no	1
Warnings:					
Information:					
3	Extension of Time	S186070000US01-EXT-ERG.pdf	23281 671c677b2308613979e88dfd57f9337d365c11a9	no	1
Warnings:					
Information:					
4	Request for Continued Examination (RCE)	S186070000US01-RCE-ERG.pdf	26872 650ab7c2af6ddf474f5ec3a16b5e63374f53d8de	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
5		S186070000US01-114AMN-ERG.pdf	128926 9a3b66f1ad618d6c3607535f4e41168ec36af8c3	yes	29

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment Submitted/Entered with Filing of CPA/RCE	1	1
	Claims	2	9

	Applicant Arguments/Remarks Made in an Amendment		10		22
	Claims		23		29
Warnings:					
Information:					
6	Affidavit-traversing rejectns or objectns rule 132	S186070000US01-DEC-ERG.pdf	1622098 ced765d316703cbb3f6748e2213be45bb5a69d15	no	24
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	32684 41bbe84e12f00cfb8c1bcbe8886cce64dc042ae6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1890295		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	R. A. Davis
Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

- [X] Fee Transmittal Form
  - [ ] Fee Attached
- [X] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [X] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) (please Identify below):

  Expert Declaration under 37 CFR 1.132

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Edward R. Gates/		
Printed name	Edward R. Gates		
Date	March 14, 2018	Reg. No.	31,616

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 14, 2018 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

6136256.1

FEE TRANSMITTAL

Complete if known	
Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Examiner Name	R. A. Davis
Art Unit	1651
Practitioner Docket No.	S1860.70000US01

[X] Applicant asserts small entity status. See 37 CFR 1.27.

[ ] Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)950.00

METHOD OF PAYMENT (check all that apply)

[ ] Check [X] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[X] Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

[ ] Charge fee(s) indicated below [ ] Charge fee(s) indicated below, **except for the filing fee**

[X] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 [X] Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
0	- 20 or HP =	0		x =	

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
0	- 3 or HP =	0		x =	

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
	- 100 =		/50 =	(round **up** to a whole number) x	=	

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 2252 Extension for response within second month	300.00
2801 Request for continued examination (RCE) - 1st ...	650.00

SUBMITTED BY

Signature	/Edward R. Gates/	Registration No. (Attorney/Agent)	31,616	Telephone	617.646.8000
Name (Print/Type)	Edward R. Gates			Date	March 14, 2018

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 14, 2018 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

6136255.1

PTO/AIA/22 (03-13)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) S1860.70000US01
Application Number 14/759,895-Conf. #3358	Filed July 8, 2015
For DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	
Art Unit 1651	Examiner R. A. Davis

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☒	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$ 300.00
☐	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☒ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

☐ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 31,616 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Edward R. Gates/ — Signature
March 14, 2018 — Date

Edward R. Gates — Typed or printed name
617.646.8000 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **03/14/2018**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 58	Minus	** 59	= 0	X $50 =	0
Independent (37 CFR 1.16(h))	* 11	Minus	***11	= 0	X $230 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

SLIE
APRIL WALKER

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	1	of	1	GROUP ART UNIT: 1651	EXAMINER: R. A. Davis

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		WO	2008/129997	A1	School Juridical Person Kitasato Gakuen	10-30-2010	Y

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		SATOH et al., Collagen reconstitution is inversely correlated with induction of limb regeneration in Ambystoma mexicanum. Zoolog Sci. 2012 Mar;29(3):191-7. doi: 10.2108/zsj.29.191.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

DOCKET NO.: S1860.70000US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	R. A. Davis
Art Unit:	1651

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Dated: April 4, 2018 Electronic Signature for Chelsie Bailey: /Chelsie Bailey/

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed before the mailing of a first Office Action after the filing of a Request for Continued Examination under 37 C.F.R. § 1.114.

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Kevin MacDonald/_____
Kevin MacDonald, PhD, Reg. No. 74,522
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: S1860.70000US01
Date: April 4, 2018

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ZOOLOGICAL SCIENCE 29: 191–197 (2012)

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Collagen Reconstitution Is Inversely Correlated with Induction of Limb Regeneration in *Ambystoma mexicanum*

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Amphibians can regenerate missing body parts, including limbs. The regulation of collagen has been considered to be important in limb regeneration. Collagen deposition is suppressed during limb regeneration, so we investigated collagen deposition and apical epithelial cap (AEC) formation during axolotl limb regeneration. The accessory limb model (ALM) has been developed as an alternative model for studying limb regeneration. Using this model, we investigated the relationship between nerves, epidermis, and collagen deposition. We found that *Sp-9*, an AEC marker gene, was upregulated by direct interaction between nerves and epidermis. However, collagen deposition hindered this interaction, and resulted in the failure of limb regeneration. During wound healing, an increase in deposition of collagen caused a decrease in the blastema induction rate in ALM. Wound healing and limb regeneration are alternate processes.

Key words: limb regeneration, epidermis, nerve, accessory limb model, *Sp-9*, collagen

INTRODUCTION

Urodele amphibians have powerful regenerative capabilities, as they can regenerate missing body parts, including limbs. Limb regeneration begins immediately after amputation. The surrounding epidermal cells migrate and cover the amputation surface (Carlson et al., 1998). An interaction between the nerves and epidermal cells covering the amputation surface then occurs, resulting in the induction of a functional epithelial tissue called the apical epithelial cap (AEC). In the presence of nerves and AEC, undifferentiated cells called blastema cells emerge from differentiated tissues (Satoh et al., 2007; Satoh et al., 2010a). These blastema cells accumulate around the amputation plane and form a regeneration blastema that undergoes limb developmental processes to restore the original structure (Bryant et al., 2002; Gardiner et al., 2002). Although the mechanisms of limb regeneration are beginning to be understood, the induction process of blastema cells has not been well elucidated. Undifferentiated blastema cells are induced from differentiated tissues by a process called dedifferentiation. Dedifferentiation is a key event during the early phases of limb regeneration, highlighting the need to further investigate the early phases of limb regeneration.

Nerves are essential for limb regeneration, and neurogenic limbs become nonregenerative when nerves are removed (Wallace, 1981). In addition, denervation during an early phase of limb regeneration terminates limb regeneration, even when in progress (Singer, 1952). Thus, nerves play an important role in limb regeneration. The effects of nerves on limb regeneration have been investigated, and some factors, such as the nerve trophic factor, have been reported (Brockes and Kintner, 1986; Mullen et al., 1996; Wang et al., 2000; Dungan et al., 2002; Nye et al., 2003; Satoh et al., 2008b). Furthermore, a focus has been on these factors and the presumptive effects of nerves on the blastema mesenchyme. However, the neural regulation of the blastema epithelium has not been well studied. Recently, a new study model—the accessory limb model (ALM)—was established in a limb regeneration study (Endo et al., 2004). This model reveals an interaction between the nerves and wound epithelium during the early phases of limb regeneration (Satoh et al., 2008b; Satoh et al., 2009). Fibroblast growth factors (FGFs) are secreted by nerves and received by the overlying epithelium (Satoh et al., 2008b). FGF-exposed epithelium is specified as AEC, an essential component in limb regeneration, because its removal results in the failure of limb regeneration (Thornton, 1957; Wallace, 1981). Undifferentiated blastema cells are induced in the presence of AEC and nerves (Satoh et al., 2008a; Satoh et al., 2010a). The induced blastema cells are also maintained by the nerves and AEC. If nerves are eliminated from a regenerating limb, the blastema stops growing and starts differentiating or regressing, depending on the phase of limb regeneration (Singer, 1952).

As described above, ALM is an alternative model for limb regeneration studies and is suitable for studying the involvement of nerves in limb regeneration. Only nerve deviation and skin wounding are enough to induce a completely patterned limb without limb amputation (Endo et al., 2004). Skin wounding without nerve deviation results in simple skin regeneration in axolotl. The induced limb is called an acces-

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doi:10.2108/zsj.29.191

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sory limb. An axolotl cannot move the accessory limb, although it has the same histology as a regular limb. Furthermore, during the process of limb regeneration in ALM, the same gene expression pattern can be seen as during regular limb regeneration and development (Satoh et al., 2007; Satoh et al., 2008b; Satoh et al., 2009). Moreover, the induced ALM blastema shows the same features as a regular blastema grown on an amputated limb (Satoh et al., 2007; Satoh et al., 2010a). ALM has several advantages compared to the regular limb regeneration model using the amputated limb, such as easier comparison of wound healing and limb regeneration. In an amputated limb, limb regeneration responses begin after amputation. When wound healing is needed for comparison, repeated denervation treatment appears to be necessary to inhibit limb regeneration and induce wound healing (Petrosky et al., 1980). Furthermore, the tissue types at the amputation surface are diverse, and each tissue drives its own healing process. Thus, wound healing in the regular model is not simple. In contrast, in ALM, only skin wounding and nerve deviation are sufficient to induce the blastema. Wounding without nerve deviation results in skin healing, without any damage to the deeper tissues such as muscles (Endo et al., 2004). Thus, using this model, comparison of wound healing and regeneration becomes easier. Furthermore, a completely patterned limb in ALM is reconstituted by a single regeneration system (AEC-dependent regeneration), while that in a regular limb regeneration model requires two different types of regeneration systems (AEC-dependent and -independent regeneration) (Satoh et al., 2010a). The major feature of ALM is limited tissue contribution. Thus, ALM reduces the complexity of limb regeneration.

In this study, we used ALM and focused on the involvement of nerves in AEC induction as well as the inhibitory effect of collagen deposition, which is synthesized during wound healing. Our results suggest that the regulation of collagen deposition by AEC and the nerves is important during limb regeneration.

MATERIALS AND METHODS

Animals and surgery

Animals that were 10–15 cm (nose to tail) in length were obtained from personal breeders and raised at 22°C. For ALM surgery, animals were anesthetized with MS-222. After surgery, they were put on ice in order to attach the rerouted nerve to the limb tissue. Regular limb regeneration surgery was performed according to a previously described protocol with some modifications (Endo et al., 2004). In brief, to reroute the nerve under intact skin, we first pulled the nerve out from a small incision made in the dorsal region of the upper arm. Then, a second small incision was made in the anteroventral region. A small tunnel was made with a fine forceps, and the nerve was pulled out through the tunnel from the dorsal to anteroventral region. The excess length of the nerve over the incision was cut, providing tension to the nerve. Because of this tension, the nerve was slightly smaller than normal. As a result, the nerve ending was located in the anterior region. The anterior epidermis was scraped with a syringe needle (27G; Terumo) to reroute the nerve to the skin collagen layer, and the nerve was pulled through the small incision. The nerve was subsequently placed on the exposed collagen. For nerve rerouting on specific days after skin wounding, a skin wound was first created on the anterior side of the upper limb, and the nerve was rerouted as described above. The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, and then incubated in water with 0.01% Tween 20, FMSF (1.74 μg/ml), and glutaraldehyde (20.18 μg/ml) for two days, resulting in an acellular ECM sheet. The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 37°C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed on the rerouted nerve.

Histology and immunohistochemistry

Samples were fixed overnight in 4% paraformaldehyde diluted with PBS. Next, the samples were incubated overnight in 10% EDTA for decalcification, and then in 30% sucrose at 4°C. The samples were then embedded in OCT compound (Sakura). A cryostat (1850; Leica) was used to cut 10-μm thick sections. Histological procedures were identical to those described previously (Satoh et al., 2009b).

We used anti-acetylated tubulin (Santa Cruz) and type I collagen antibody (Sigma) to visualize axons and collagen, respectively. Antigen retrieval was performed for collagen staining by incubating the sections in 5 μg/ml proteinase K in Tris-buffered saline with 0.01% Tween 20 for 30 min at room temperature, as described previously (Satoh et al., 2008a).

RT-PCR and in situ hybridization

Total RNA was prepared using the TriPure reagent (Roche). Reverse transcription was performed with Superscript III (Invitrogen). The following primers were used for RT-PCR: Sp-9 forward, 5'-GTGTGCAACTGGCTCTTCTG-3'; Sp-9 reverse, 5'-TGCGTTTT-CATGTGTTTGCT-3' and Ribosomal protein L19 forward, CATG-GGCACTGGTAAGAGAAAAG; Ribosomal protein L19 reverse, GCGGCGCAAGATTCTCAT. In situ hybridization followed previously published procedures (Satoh et al., 2007).

RESULTS

Direct interaction between the nerves and epidermis resulted in Sp-9 activation

A previous study of ALM revealed that an interaction between the nerves and wound epithelium is important for *Sp-9* activation (Satoh et al., 2008b). However, it was unclear whether a physical interaction was necessary to induce *Sp-9* expression. To investigate this, we deviated nerves under or on the dermal collagen layer (Fig. 1). When nerves were deviated under the dermal collagen layer (Fig. 1A), the direct interaction between the nerves and epidermis was inhibited. Axon ends could be seen under the dermal collagen layer (Fig. 1A, asterisk), but AEC formation was not observed histologically in the overlying epidermis (Fig. 1B), suggesting that direct interaction between the nerves and epidermis is necessary to induce AEC. To investigate this further, axons were visualized by immunohistochemistry (Fig. 2). When nerves were deviated under the dermal collagen layer, axon ends were found to be located far from the overlying epidermis (Fig. 1A). Furthermore, the epidermis did not contain axons from the deviated nerves (Fig. 2A). This finding also suggests that direct contact between the axons and epidermis was inhibited. Next, to create direct interaction between the nerves and epidermis without fibroblasts, nerves were placed on the dermal collagen layer after scratching the epidermal cells (Fig. 1C). This caused the epidermal cells to migrate and surround the rerouted nerves. The surrounding epidermis became thicker than the normal epidermis. The surrounding epidermis was largely dedifferentiated, as no Leydig cells, which are one type of

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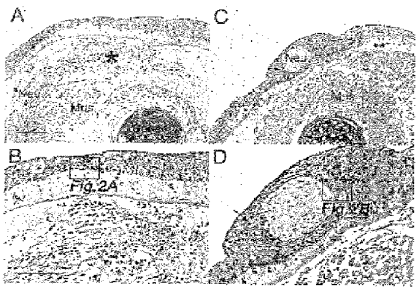


Fig. 1. Nerve deviation under or on the dermal collagen layer. **(A, B)** A nerve was deviated under the collagen layer. The nerve ends were seen under the dermal collagen layer (A, asterisk). Panel B is a higher magnification of panel A. **(C, D)** A nerve was deviated on the dermal collagen layer. The nerve was surrounded by the epidermis, which appeared to be undifferentiated. Panel (D) is a higher magnification of C. Sections were made 72 h after surgery and stained with Alcian blue and hematoxylin and eosin. Mus, muscle; Neu, neural tissues. Scale bar in (A) = 200 μm. Scale bar in C = 100 μm. (A) and (C) are the same magnification as (B) and (D). Arrow in (D) indicates a Leydig cell in the epidermis.

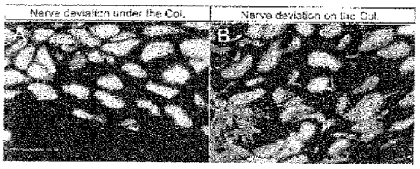


Fig. 2. Visualization of axon fibers. Axon fibers were stained with anti-acetylated tubulin antibody (red) and nuclei with DAPI (blue). **(A)** The deviated nerve under the dermal collagen layer did not reach the axons in the overlying epidermis. **(B)** Axon fibers could be seen in the epidermis. The deviated nerve extended its axons into the epidermis. A and B are the adjacent section of the boxed area in Fig. 1B and 1D, respectively. Scale bar in A = 50 μm. A and B are the same magnification.

secretory cell in axolotl skin (Fig. 1D, arrow), were present around nerves (Fig. 1D). In the surrounding epidermis, newly extended axon fibers could be seen (Fig. 2B). We then investigated *Sp-9* induction by the direct interaction between the nerves and epidermis. *Sp-9* was upregulated in samples in which nerves were deviated on the dermal collagen layer and was absent in normal skin (Fig. 3A). Consistent with histological observations, *Sp-9* upregulation was not detected in samples in which nerves were deviated under the collagen layer. Furthermore, we examined *Sp-9* expression by in situ hybridization (Fig. 3D, E) and detected *Sp-9* throughout the epidermis where axon fibers were detectable (Fig. 3B–D). Normal epidermis in the same section did not show intense signals for *Sp-9* expression (Fig. 3E). These results suggest that *Sp-9* expression is activated by the direct interaction between the nerves and epidermis, and AEC formation is induced by this direct interaction.

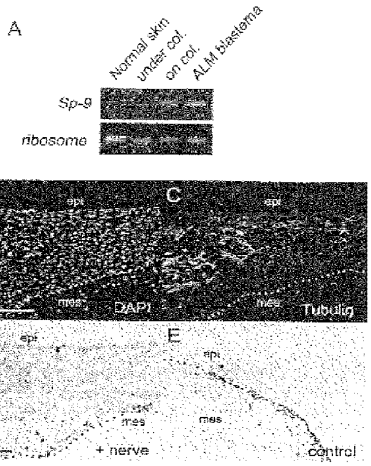


Fig. 3. *Sp-9* expression by nerve deviation. **(A)** RT-PCT analysis. *Sp-9* was not detected in normal skin. The rerouted nerve under the collagen layer (lane 2) did not activate *Sp-9* expression. In contrast, the deviated nerve on the collagen layer (lane 3) increased *Sp-9* expression. The ALM blastema also showed *Sp-9* expression. **(B–E)** Sections of the epidermis 7 days after nerve deviation. (B, C) Axons were visualized as green signals and nuclei as blue. Axons could be seen in the epidermis. (D) The *Sp-9* expression pattern was revealed by in situ hybridization shown in the adjacent sections of (B) and (C). (E) In the normal skin region of the same section as (D), no *Sp-9* signal was detected. Scale bar in B = 300 μm. Scale bar in D = 100 μm. (B) and (D) are the same magnification as (C) and (E). Dotted lines in (B–E) indicate the border of the epidermis. The upper side is apical, and the bottom side is basal. Epi. = epidermis. Mes. = mesenchyme (dermis and muscle).

Blastema induction rate decreased as wound healing progressed

The collagen layer in the dermis inhibited the direct interaction between the nerves and epidermis, resulting in the failure of AEC induction. In axolotl, collagen is reorganized during wound healing (Endo et al., 2004; Satoh et al., 2008a). We hypothesized that the AEC induction rate in ALM would decrease as collagen accumulated during skin wounding. To examine this, we created a wound, rerouted nerves on specific days after skin wounding, and investigated blastema formation. As expected, a blastema was seen after skin wounding each time the nerve was deviated (regular ALM surgery, data not shown). The blastema induc-

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tion rate remained high when nerves were rerouted in 3-day-old wounds (Table 1, Fig. 4A, B); the wound healing region remained transparent three days after wounding (Fig. 4A). The blastema induction rate decreased in 5-day-old wounds (Table 1, Fig. 4C, D), and almost half of the examined limb grew a blastema (Table 1). At five days, the wound became slightly opaque (Fig. 4C), and at 10 days, the wound became more opaque and blastema induction was completely inhibited (Table 1, Fig. 4E, F). Pigment cells migrated into the 15-day-old wounds, suggesting that dermal tissue had regenerated (Fig. 4G) and that blastema induction was no longer possible (Table 1, Fig. 4G, H). We also investigated *Sp-9* expression by RT-PCR (Fig. 4I). *Sp-9* expression was consistently detectable in samples in which blastemas were formed. Very faint *Sp-9* expression was detectable in 10- and 15-day wounds in which nerves were deviated. We investigated collagen deposition by immunohistochemistry. Three- and 5-day-old wounds did not show much deposition (Fig. 5A–D), although we began to detect signals of type I collagen by day 5 (Fig. 5E–H). Collagen fibers were resynthesized in 10-day-old wounds (Fig. 5I–L), which was consistent with a previous study (Satoh et al., 2008a). Additional collagen fibers were deposited and organized in 15-day-old wounds (Fig. 5M–P). These results suggest that collagen deposition decreased the blastema induction rate.

Table 1. Blastema induction rate.

	Induction of ectopic blastema	No induction of ectopic blastema	Sum
Nerve deviation at day 0	10 (100%)	0 (0%)	10
Nerve deviation at day 3	11 (91.7%)	1 (8.3%)	12
Nerve deviation at day 5	13 (52%)	12 (48%)	25
Nerve deviation at day 10	0 (0%)	19 (100%)	19
Nerve deviation at day 15	0 (0%)	11 (100%)	11

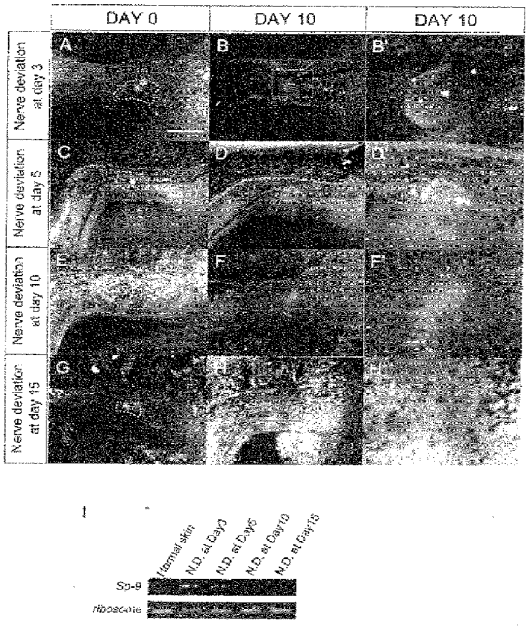


Fig. 4. Nerve deviation to wounds on specific days after wounding. (A–H) Anterior view of the limb with the rerouted nerve immediately (A, C, E, G) and 10 days (B, D, F, H) after surgery. Wound margin and putative wound margin were outlined by the dotted line in (A), (C), (E), and (G). Asterisks indicate the deviated nerve ends. (B'), (D'), (F'), and (H') are the higher magnification views of the boxed region of (B), (D), (F), and (H), respectively. Arrows in B and D indicate the growing bump. All panels in (A–H) show heads to the right. Scale bar in A = 3 mm. (A–H) are the same magnification. (I) RT-PCR analysis showed that *Sp-9* was expressed. All samples were harvested 10 days after nerve deviation.

Placing an acellular collagen sheet on the deviated nerves inhibited blastema formation

To investigate whether collagen was related to the inhibition of blastema induction, we placed a collagen sheet on the deviated nerves at the same time as ALM surgery. An acellular collagen sheet was prepared as described in the Materials and Methods section. To confirm the absence of living cells, we prepared a section of the collagen sheet (Fig. 6A). The section was stained with hematoxylin to visualize the nuclei. However, nuclei were not detectable in this sheet, indicating only collagen bundles and no living cells. Next, a black axolotl-derived collagen sheet was labeled with green fluorescent dye and placed on the rerouted nerves (Fig. 6B). Ten days after surgery, no apparent blastema formation was observed (Fig. 6C). To confirm this, we cut sections (Fig. 6D–F) and found that the wound epithelium migrated over the collagen sheet (Fig. 6D). AEC was generally thickened; however, the wound epithelium that had migrated over the collagen layer was not thickened, suggesting no AEC formation. No axons were observed in the overlying epidermis (Fig. 6E). To further investigate this process, we performed in situ hybridization of the sections. *Prrx-1*, a blastema marker gene, was not detected (Fig. 6F), while the regular ALM blastema expressed *Prrx-1* in the absence of the collagen sheet (Fig. 6G). Next, we changed the nerve position in a similar experiment (Fig. 7). In this case, we rerouted the nerve on the grafted collagen sheet (Fig. 7A, B). Ten days after the surgery, the blastema

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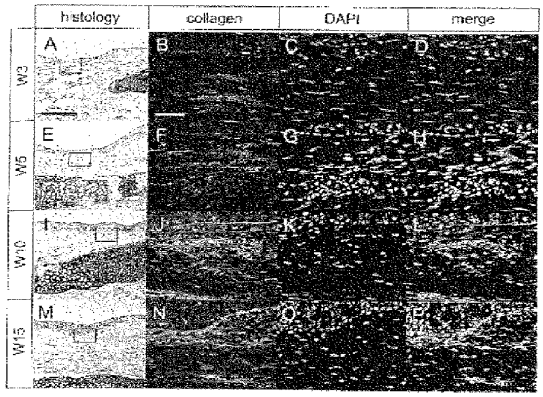
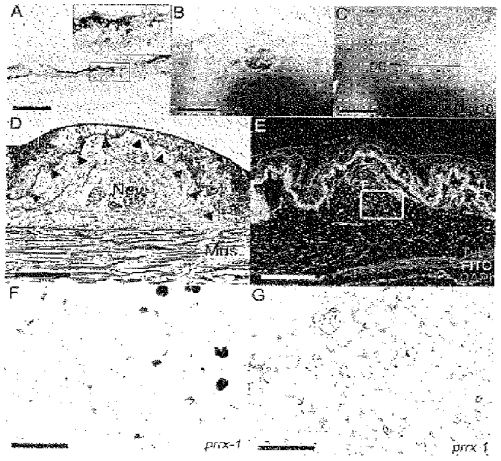


Fig. 5. Collagen reorganization during wound healing. **(A–D)** Three-day-old wound. **(E–H)** Five-day-old wound. **(I–L)** Ten-day-old wound. **(M–P)** Fifteen-day-old wound. (A, E, I, M) Alcian blue and hematoxylin and eosin staining. (B, F, J, N) Collagen was visualized by immunohistochemistry (green). (C, G, K, O) Nuclei stained with DAPI (blue). (D, H, L, P) Merged images. (A–D) Collagen deposition was not observed in the 3-day-old wound. B and C are higher magnification images of the boxed region in A. (E–H) Collagen synthesis was detected in the 5-day-old wound. (F–H) are higher magnification images of the boxed region in E. (I–P) Collagen bundles were observed late. (J–L) and (N–P) are higher magnification images of the boxed region in I and M, respectively. Scale bar in (A) = 0.7 mm. Scale bar in (B) = 100 μm. (A), (E), (I), and (M) are at the same magnification. The remaining images are of the same magnification as (B).



was observed (Fig. 7C, D). To confirm the identity of the blastema, sections were made. The grafted collagen was stained with green fluorescent dye as mentioned (Fig. 7E, G). The morphology of the collagen sheet was disrupted, suggesting that the grafted collagen sheet seemed to be partially digested by endogenous enzymes because a blastema shows higher MMP activities (Yang et al., 1999). *Prrx-1*-positive cells were accumulated between the collagen sheet and blastema epithelium (Fig. 7H, arrows). These data strongly suggest that blastema formation can be induced by a deviated nerve on a collagen sheet. Taken together with the inhibitory effect of a collagen sheet for blastema formation indicated in Fig. 6, our results suggest that collagen acts as a barrier for interaction between nerves and the epidermis.

DISCUSSION

Nerve function during AEC induction

Nerves are necessary for limb regeneration, and ALM provides many insights into nerve function during limb regeneration. Previous ALM studies have suggested that nerves play important roles in AEC induction, leading to blastema formation (Endo et al., 2004; Satoh et al., 2007; Satoh et al., 2008b; Satoh et al., 2009; Satoh et al., 2010a). FGFs are secreted from nerves during axolotl limb regeneration and may induce AEC formation (Satoh et al., 2008b). *Sp*-9 is probably activated by nerve-secreted FGFs at the early phase of limb regeneration. In the case of limb development in higher vertebrates, *Sp*-9 is also FGF-dependent (Kawakami et

Fig. 6. An acellular collagen sheet inhibited blastema induction. (A) A section of the prepared collagen sheet showing the absence of living cells. Insert shows higher magnification of the boxed region. **(B)** Green fluorescent-labeled collagen was placed on the deviated nerve. **(C)** Collagen sheet grafting did not result in blastema formation 10 days after surgery. **(D–F)** Sections of the collagen sheet-grafted and nerve-deviated wounds are indicated by the line in C. (D) The section was stained with hematoxylin and eosin. Arrowheads indicate the collagen sheet. Nev. = nerves. Mus. = muscles. (E) The collagen sheet was labeled with green fluorescent dye, and axons were visualized by immunohistochemistry (red). *Prrx-1* expression was investigated by in situ hybridization in the collagen sheet-grafted sample (F) and ALM blastema harvested 10 days after ALM surgery (G). Scale bar in B, D, and F are 2 mm, 500 μm, and 100 μm, respectively. E and G are the same magnification as D and F, respectively.

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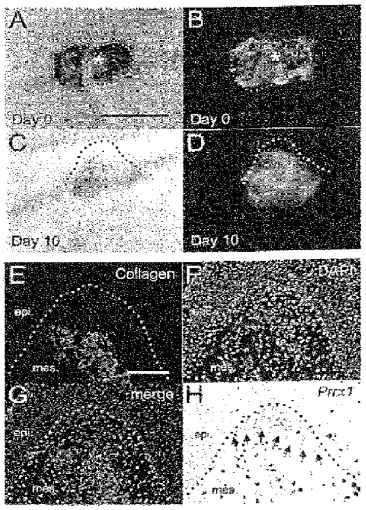


Fig. 7. Collagen sheet graft below the rerouted nerve did not inhibit blastema induction. Anterior view of the limb with the rerouted nerve immediately **(A, B)** and 10 days **(C, D)** after surgery. Asterisks in (A) and (B) indicate the end of the rerouted nerve. The grafted collagen was stained with green fluorescent dye as described in the Materials and Methods section. Dotted lines in (A) and (B) outline the wound margin. Dotted lines in (C) and (D) outline the induced bump. **(E–H)** Sections of the bump with collagen sheet. (E) The grafted collagen was detectable because it was stained with green fluorescent dye. (F) Nuclei were visualized by DAPI. (G) Merged image. Cells were accumulating between the grafted collagen sheet and the blastema epithelium. Such population was *Prrx-1*-positive (H, arrow). Epi. = blastema epithelium. Mes. = blastema mesenchyme. Scale bars in A and E are 2 mm and 400 μm, respectively. (A–D) are at the same magnification. (E–H) are at the same magnification.

al., 2004). This is not unexpected, as most limb regeneration processes are similar to those of limb development. The initial *Sp-9* expression pattern spreads throughout the wound epithelium and is then restricted to the basal layer (Satoh et al., 2008b; Fig. 3D). This finding suggests that the interaction of the blastema mesenchyme with the basal layer of the epidermis would be necessary for a restricted *Sp-9* expression pattern. Although a mesenchymal contribution may be necessary for a completely functional AEC, the nerves and epidermis are sufficient for initial *Sp-9* expression (Figs. 1–3). However, we believe that another issue remains unresolved. In our study, epidermal cells migrated, and therefore, we could not determine whether nonmigratory epidermal cells respond to neural factors. This question will be addressed in a future study.

Collagen deposition hinders blastema induction

The dermal collagen layer acts as a barrier for axon penetration (Figs. 4–6). However, a few axons were evident in the dermal layer. Hence, dermal collagen may not inhibit axon penetration completely but may make it difficult, which is consistent with the results shown in Figs. 4 and 5. It became difficult to induce a blastema due to collagen deposition (Table 1, Fig. 4). Deposition of collagen began to reconstitute dermal tissues after wounding without nerve deviation, a process that occurs during regular wound healing. Urodele amphibians can restore skin damage without scarring (Levesque et al., 2010), although collagen deposition occurs (Endo et al., 2004, Fig. 5). In higher vertebrates, collagen reconstitution occurs after skin wounding. Such reconstituted collagens, however, are not usually re-organized well, resulting in scar formation (Gurtner et al., 2008). The reason why urodele amphibians is able to undergo a scarless healing process has remained a mystery. To understand why, it would be helpful to describe collagen deposition processes in axolotl skin damage. Obvious collagen synthesis could not be detected in 3-day-old wounds (Fig. 5A–D). Epidermal cells maintain the ability to receive neural inputs at this time. However, 5-day-old wounds showed a decreased ability to form AEC (Table 1). Simultaneously, collagen deposition appeared to have just begun because faint collagen signals were observed by immunohistochemistry (Fig. 5E–H). Otherwise, epidermal cells begin losing their ability to receive neural inputs. In this case, the reason why epidermal cells start losing this ability needs to be determined. Finally, the blastema induction rate decreased to zero (Table 1, Fig. 5I–P), and collagen bundles were observed (Fig. 5J, N). Three possibilities account for the inhibition of blastema formation. First, reorganizing collagen acts as a physical barrier that blocks axon penetration. Because axons cannot penetrate through the collagen, the epidermal cells cannot activate *Sp-9* due to the lack of sufficient neural inputs. Second, as mentioned above, epidermal cells lose their ability to receive neural inputs. Third, reorganizing collagen absorbs neural factors, including FGFs. In fact, the extracellular matrix (ECM) traps FGF4 during chicken limb development (Schaller and Muneoka, 2001), and FGFs also bind well to ECM (Powers et al., 2000). Hence, newly synthesized collagen may absorb FGFs from nerves. Perhaps all of these factors are important for the early reactions during limb regeneration. A previous study showed that wound healing (collagen deposition) is inhibited on induction of AEC formation (Satoh et al., 2008a; Satoh et al., 2010a). Collagen deposition remains inhibited in a blastema environment. The blastema has many axon fibers, and the distal AEC is richly innervated (Satoh et al., 2008b; Thornton, 1956; Singer, 1949). Furthermore, the blastema is rich in FGFs secreted by AEC, nerves, and the blastema mesenchyme (Endo et al., 2000; Han et al., 2001; Yokoyama et al., 2001; Dungan et al., 2002; Satoh et al., 2008b). Thus, maintaining a lower level of collagen deposition may be important for the distal blastema environment. The opposite phenotype is observed between the blastema and wound healing during collagen deposition (dermal differentiation). Collagen function during limb regeneration should be studied more closely to allow a more detailed understanding of limb regeneration.

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ACKNOWLEDGMENTS

This study was supported by the Naito Foundation and Special Coordination Funds for Promoting Sciences and Technology of the Ministry of Education, Sports, Culture, Science and Technology of Japan.

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(Received July 1, 2011 / Accepted September 27, 2011)

Publication number: **WO08129997 A1**

Publication country: WIPO (P.C.T)

Publication type: APPLICATION WITH SEARCH REPORT

Publication date: 20081030

Application number: WO2008JP57427

Application date: 20080416

Priority: JP20070108077 20070417 JP20070108077;

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International class[8]: C12N5/0775;

Cooperative Patent class: C12N5/0068 ; C12N5/0663 ; C12N2533/92 ;

Designated states: AE AG AL AM AO AT AU AZ BA BB BE BF BG BH BJ BR BW BY BZ CA CF CG CH CI CM CN CO CR CU CY CZ DE DK DM DO DZ EC EE EG ES FI FR GA GB GD GE GH GM GN GQ GR GT GW HN HR HU ID IE IL IN IS IT JP KE KG KM KN KP KR KZ LA LC LK LR LS LT LU LV LY MA MC MD ME MG MK ML MN MR MT MW MX MY MZ NA NE NG NI NL NO NZ OM PG PH PL PT RO RS RU SC SD SE SG SI SK SL SM SN SV SY SZ TD TG TJ TM TN TR TT TZ UA UG US UZ VC VN ZA ZM ZW

Family members: DE602008020608 D1 EP2149601 A1 EP2149601 A4 EP2149601 B1 JP2008129997 A1 JP5458279 B1 JP5458279 B2 WO08129997 A1

Title: COMPOSITION OF SOLUBILIZED AMNION AND USE THEREOF
COMPOSITION D'AMNIOS SOLUBILISEE ET UTILISATION DE CETTE COMPOSITION

Title: 可溶化羊膜組成物及びその用途

Abstract:

Disclosed are an inexpensive composition of amnion, which is more excellent in handleability and more suitable for cell culture than a culture dish coated with the amnion itself; and a coated material using the same, particularly a safe coated material for cell culture, which is suitable for regenerative medicine such as stem cells. The composition of amnion contains a solubilized substance of amnion, which is solubilized by an acid treatment under heating, in a vehicle. The coated material is obtained by coating a substrate with the composition of amnion followed by drying. Accordingly, a safe and inexpensive material, which is more excellent in handleability and more suitable for cell culture than the amnion itself is provided, and by using the material, the growth potential of cells can be increased.

L'invention concerne une composition economique d'amnios, qui presente des qualites superieures de maniabilite et qui est plus adaptee a la culture cellulaire qu'une boite de Petri enduite de l'amnios en tant que tel; et un materiau d'enduction utilisant ladite composition, en particulier un materiau d'enduction sur pour la culture cellulaire qui est adapte pour la

medecine regenerative, par exemple pour des cellules souches. La composition d'amnios contient une substance d'amnios solubilisee qui est solubilisee, dans un vehicule, par traitement a l'acide dans des conditions de chauffage. Le materiau d'enduction est obtenu en enduisant un substrat de la composition d'amnios, puis en le sechant. On obtient de la sorte un materiau sur et economique, qui presente des qualites superieures de maniabilite et qui est plus adapte a la culture cellulaire que l'amnios lui-meme. En outre, en utilisant la substance, on peut augmenter le potentiel de croissance de cellules.

Abstract:

羊膜自体をコーティングした培養ディッシュよりも取り扱い性に優れ、より細胞培養に適した安価な羊膜組成物およびそれを用いたコーティング材、特に幹細胞などの再生医療に適した安全な細胞培養用コーティング材が開示されている。該羊膜組成物は、加熱下での酸処理により可溶化した羊膜の可溶化物を媒体中に含む。また、該コーティング材は、該羊膜組成物を基体上にコーティングし、乾燥して成る。これらにより、羊膜自体よりも取り扱い性に優れ、細胞の培養に適した安全で安価な材料を提供し、該材料を用いることで細胞の増殖能を高めることが可能である。

The excellent handling properties than the culture dish that was coated

amnion itself, cheap amnion composition suitable for cell culture and more and coating material using the same, suitable for regenerative medicine such as stem cells, particularly Secure cell culture coating material is disclosed.?? film composition comprises a medium lysates amniotic membrane was solubilized by acid treatment under heat. Further, the coating on a substrate ?? film composition, the coating material is formed by drying. It is possible to increase the proliferative capacity of the cells by result, excellent handling properties than the amnion itself, to provide a material safe and inexpensive suitable for culturing cells, the use of material.

Claims:

[1] 加熱下での酸処理により可溶化した羊膜の可溶化物を媒体中に含む可溶化羊膜組成物。

[2] 前記酸が酢酸である請求項1記載の組成物。

[3] 塩基で中和して中性のpHを有する請求項1又は2記載の組成物。

[4] 前記塩基がアンモニア水である請求項3記載の組成物。

[5] 媒体が水である請求項1ないし4のいずれか1項に記載の組成物。

[6] 前記羊膜がヒト由来である請求項1ないし5のいずれか1項に記載の組成物。

Solubilized amnion composition comprising medium lysates of amniotic membrane was solubilized by acid treatment

in [1] under heating.

The composition according to claim 1, wherein the acid

[2] the acid.

composition according to claim 1 having a neutral pH by neutralization

[3] base.

The composition of claim 3, wherein the aqueous ammonia

[4] said base.

claims 1 is water

[7] 請求項1ないし6のいずれか1項に記載の可溶化羊膜組成物を基体上にコーティングし、乾燥して成る可溶化羊膜コーティング材。

[8] 請求項7記載の可溶化羊膜コーティング材から成る細胞培養用基材。

[9] 前記細胞が間葉細胞である請求項8記載の基材。

[10] 前記間葉細胞がヒト羊膜由来である請求項9記載の基材。

[11] 前記細胞が間葉系幹細胞である請求項8記載の基材。

[12] 前記間葉系幹細胞がヒト骨髄由来である請求項11記載の基材。

[13] 請求項7記載の可溶化羊膜コーティング材上で細胞を培養することを含む細胞培養方法。

[5] the medium composition according to any one of claims 4.

claims 1 derived from human

[6] The amniotic membrane composition according to any one of claims 5. not [7] claims 1 to solubilized amnion coating material is coated on a substrate solubilized amnion composition according to any one of claims 6, it is obtained by drying.

cell culture substrate made from sheep solubilized membrane coating material 7, wherein

[8] claim.

substrate according to claim 8, wherein the mesenchymal cell

[9] the cell.

substrate according to claim 9, wherein a human amnion-derived

[10] the mesenchymal cells.

substrate according to claim 8, wherein the mesenchymal stem cell is

[11] the cell.

base material according to claim 11, wherein a human bone marrow-derived

[12] the mesenchymal stem cells.

cell culture method comprising culturing cells in soluble amnion coating material onto 7 wherein

[13] claims.

Description:

[0001] 本発明は、可溶化羊膜組成物及びその細胞培養用基材の原料としての用途に関する。

[0002] 細胞を基体上で培養する細胞培養において、細胞の増殖速度を大きくするために、プラスチック製やガラス製の基体にコラーゲンやフィブロネクチンをコーティングして培養基材とし、この培養基材上で細胞を培養することが知られてい

[0001] The present invention relates to the use of as a raw material for the cell culture substrate and solubilized amnion composition. In cell cultures cultured on a substrate

The

[0002] cells, in order to increase the growth rate of the cells, the culture substrate by coating the fibronectin or collagen substrate of glass or plastic, the culture medium by culturing cells on materials

る。そして、培養基材に用いるコラーゲンとして、羊膜から採取したコラーゲンを用いることも公知である（非特許文献１）。また、細胞を除去した羊膜自体を培養基材に用いることも知られている（非特許文献２及び３）。

[0003] 非特許文献 1: T. R. KUMAR, et al., J. Biomater. Sci. Polymer Edn, Vol. 14, No. 7, pp. 689-706 (2003) 非特許文献 2: Morio Ueno et al., PNAS, vol.103, no.25, 9554-9559, June 20, 2006

発明の開示

発明が解決しようとする課題

[0004] 羊膜自体を培養基材として用いる場合、取扱い性や保存性に問題がある。すなわち、細胞の培養は、培養ディッシュやマイクロプレートのウエル中等で行なわれることが多いが、その場合には、培養ディッシュやウェルの形状に合うように羊膜を裁断しなければならず、また、羊膜が基体からめくれ上がる恐れもある。特に、細胞を長期間培養する場合には、細胞が培養ディッシュ底面に接着した状態のまま培養液を交換する必要があり、この培養液の交換に伴う液体の流動が物理的な衝撃となり、羊膜自体が基体からめくれあがり、培養実験が中止される問題がある。

[0005] フィブロネクチンやコラーゲンなどの他のコーティング材は、その組成物の調製工程において種々の酵素によって処理されており、これらの酵素もヒト以外の生物に由来するもの、又はヒトの遺伝情報に基づいたアミノ酸配列を有する酵素であっても大腸菌を用いた組換えタンパク質が多いため、これら酵素等の物質がコーティング材に残留することは、該コーティング材を用いて培養された細胞を細胞移植治療に用いる場合や、他の移植治療に用いる細胞と共培養する場合などに深刻な問題を残す。

[0006] また、フィブロネクチンやコラーゲンなどの他のコーティング材は、その生成物の調製過程で酵素処理を行うが、一般に酵素は精製が困難で

is known. Further, known also as the collagen to be used for the culture substrate is used collagen taken from amniotic membrane (Non-Patent Document 1). Further, (3 and Non-Patent Document 2) to be used for the culture substrate amnion itself to remove cells are also known.

1

[0003] Non-Patent Document:... TR KUMAR, et al, J. Biomater Sci Polymer Edn, 2 689-706 (2003) et al Vol 14, No 7, pp: Morio Ueno... disclosure et al., PNAS, vol.103, no.25, 9554-9559, of the 20, 2006

invention June

problems to be resolved by the invention

When used as a culture substrate

the

[0004] The amniotic membrane itself, there is a problem in storage stability and handling properties. That is, the culture of cells is preferably performed in a well secondary microplate or culture dish is large, in which case, it also has to cut the amniotic membrane to match the shape of the well or culture dish, amniotic But there is a possibility that curling up from the substrate. In particular, in the case of long-term culture of cells, it is necessary to replace the culture medium remains the cells had attached to the culture dish bottom, the flow of liquid with the exchange of the culture medium is a physical shock, amniotic itself Mekurea rising from the base, there is a problem that culture experiment is stopped.

It has been processed by various enzymes in the preparation process of the composition, that these enzymes also is derived from non-human organisms, or coating material of others, such as collagen and

[0005] fibronectin

The inheritance of human Since the recombinant protein using E. coli may be an enzyme having the amino acid sequence based on the information, the more a substance such as an enzyme they remain in the coating material, cells cells are cultured with the coating material leaving a serious problem, for example, for co-culture or when used for transplantation therapy, and the cells used for transplantation therapy other.

あり、また市販品を入手できても高価である場合が多く、調製工程の前段階として酵素の準備が容易でないという問題がある。

[0007] 従って、本発明の目的は、羊膜自体よりも取扱い性に優れ、細胞の培養に適した安全で安価な材料を提供することである。 課題を解決するための手段

[0008] 本願発明者らは、もし、羊膜を可溶化することができれば、その可溶化物を基体上にコーティングし、乾燥させたものが培養基材として利用可能かもしれないことに想到した。この場合には、可溶化物を基体上に塗布し、乾燥させることにより培養基材とすることができるので、取扱い性は良好である。しかしながら、羊膜の可溶化物は知られていない。本願発明者らは、鋭意研究の結果、羊膜の可溶化物の調製方法を見出し、かつ、この羊膜可溶化物を基体上にコーティングし、乾燥させたものを培養基材として利用可能であり、さらに、以下において詳細に説明するように、細胞の培養に適し、該培養基材が安価に調製できる基材であることを見出し、本発明を完成した。

[0009] すなわち、本発明は、加熱下での酸処理により可溶化した羊膜の可溶化物を媒体中に含む可溶化羊膜組成物を提供する。また、本発明は、上記本発明の可溶化羊膜組成物を基体上にコーティングし、乾燥して成る可溶化羊膜コーティング材を提供する。さらに、本発明は、上記本発明の可溶化羊膜コーティング材から成る細胞培養用基材を提供する。さらに、本発明は、上記本発明の可溶化羊膜コーティング材上で細胞を培養することを含む細胞培養方法を提供する。 発明の効果

[0010] 本発明により、羊膜を可溶化した組成物が初めて提供された。本発明の組成物を基体上にコーティングし、乾燥したものは細胞の培養基材として利用可能であり、該羊膜の可溶化組成物は液体であるので、基体表面上にコーティングし、乾

[0006] The coating material of the other such as collagen and fibronectin, performs the enzyme treatment during preparation of the product, but the enzyme is expensive and also available commercially purified Further, it is difficult in general there is a problem if it is in many cases, preparation of the enzyme is not easy as a pre-stage of the preparation process.

[0007] Accordingly, an object of the present invention is that excellent handling properties than the amnion itself, to provide a material safe and inexpensive suitable for culturing cells. Means of solving the problems

If we can be solubilized amnion,

[0008] The present inventors have found, that those coated on a substrate the lysates, and dried it may be used as a culture substrate was conceived. In this case, it is possible to the culture substrate by applying onto a substrate solubilization, and the mixture is dried, the handling property is good. However, solubilization of the amniotic membrane is not known. Results of the study, the present inventors, is available as a culture substrate that is and found a process for the preparation of lysates of amniotic membrane is coated on a substrate and the amniotic membrane lysate, dried intensive, Further, they have found that, as will be described in detail below, suitable for culturing cells, is a substrate which can be prepared at a low cost the culture substrate, thereby completing the present invention.

That

[0009] The present invention provides a solubilized amnion composition comprising medium lysates amniotic membrane was solubilized by acid treatment under heating. Further, the coating on a substrate solubilized amnion compositions of the present invention, the present invention provides a solubilized amnion coating material formed and dried. Further, the present invention provides a cell culture substrate composed of solubilized amnion coating material of the present invention. Further, the present invention provides a cell culture method comprising culturing cells in soluble amnion coating material on the present invention. Effect of the Invention by

[0010] In the present invention, a composition solubilized amnion has been provided for the first

燥するだけで培養基材とすることができ、取扱い性に優れる。

[0011] また、本発明に係るコーティング材は、安価に製造可能であり、調製工程で使用した物質の残留がないため、該コーティング材上で培養した細胞は、そのまま移植治療に用いることができ、再生医療に適している。本発明に係る可溶化羊膜コーティング材は、羊膜成分の調製工程において酸処理を行い、好ましくは塩基処理により中和を行なう工程を経るが、これらの工程で使用する酸又は塩基の多くのものは揮発性であり、加熱により完全に羊膜組成物から除去することが可能である。従って、残留物質を含まない本発明のコーティング材上で培養した細胞は、そのまま細胞移植に使用することが可能である。特に再生医療の分野では、細胞移植に用いる細胞は非常に巧妙にデザインされた細胞分化誘導法に従って目的の組織細胞へと分化させる場合が多く、また、移植するタイミングも治療の成否に大きく影響するので、移植する細胞に移植直前に何らかの処理を施すことは好ましくない。従って、培養した細胞をそのまま移植することを可能とする本発明に係るコーティング材は、再生医学・再生医療の分野において大きく貢献するものと期待される。

[0012] さらに本発明は安価な酸、又は塩基を使用するだけで組成物の調製が可能であるため、他のコーティング材に比べて、簡便に、安価に調製することができるというメリットを有する。

図面の簡単な説明

[0013] [図 1] 本発明の実施例で行った、各種培養基材上で羊膜間葉細胞を培養した際の細胞数の評価結果を示す図である。 [図 2] 本発明の実施例で行なった、異なる濃度のヒト可溶化羊膜組成物でコーティングした培養基材上でヒト骨髄由来間葉系幹細胞を培養した際の細胞数の評価結果を示す図である。 [図 3] 本発明の実施例で行なった、ヒト可溶化羊膜組成物でコーティングした培養基材上でヒト骨髄由来間葉系幹細胞を培養した際の細

time. Which is coated on a substrate a composition of the present invention, and dried is used as a culture substrate of the cell, since the liquid solubilizing composition of the amniotic membrane, is coated on the substrate surface, just dried In The may be a culture substrate, and excellent handling properties.

[0011], the coating material according to the present invention, it can be produced at low cost, because there is no residual material was used in the preparation step, cells cultured in the coating material on, use in transplantation therapy as It is possible, it is suitable for regenerative medicine. And performing an acid treatment in the preparation process of the amniotic membrane component, preferably go through a step of neutralizing with a base treatment, solubilized amnion coating material according to the present invention, many of the acid or base used in these processes It is possible that it is volatile, it is removed from the amniotic membrane composition completely by heating. Thus, cells cultured with a coating material of this invention containing no residual material can be used for cell transplantation as it is. In the field of regenerative medicine, If you want to differentiate into tissue cells of interest according to the cell differentiation induction method designed very cleverly, and also many, the timing of the transplant also a large influence on the success or failure of treatment cells used for cell transplantation in particular so, applying some processing immediately prior to implantation to cells which transplantation is not preferable. Accordingly, the coating material according to the present invention makes it possible to port as the cultured cells are expected to contribute in the field of regenerative medicine and regenerative medicine.

Since the present invention is capable of preparing the compositions inexpensive acids, or using only the base further

[0012]

, and it can be compared with the coating material of the other, conveniently, is prepared at a low cost I have a merit. A brief description of

drawing

[0013] was carried out in the embodiment of FIG. 1 the present invention, is a diagram showing the

胞数の評価結果を示す図である。 [図 4] 本発明の実施例で行った、各種培養基材上でヒト骨髄由来間葉系幹細胞を培養した際の細胞数の評価結果を示す図である。 [図 5] 本発明の実施例で行なった、各種培養基材上でヒト骨髄由来間葉系幹細胞を培養し、酵素処理による培養細胞回収の回収率を比較検討した結果を示す図である。

発明を実施するための最良の形態

[0014] 上記の通り、本発明の可溶化羊膜組成物は、羊膜を加熱下において酸処理することにより得られたものであり、液体の媒体中に羊膜の溶解物が含まれたものである。

[0015] 羊膜が由来する動物種は、哺乳動物であれば特に限定されないが、ヒト細胞又はヒトに移植若しくは投与する細胞を培養する場合には、培養細胞の増殖性及び／又は安全性の観点からヒト羊膜を原料とすることが好ましい。羊膜は、出産後に胎盤と共に排出され、ヒト羊膜でも医療廃棄物として廃棄されている。従って、ヒト羊膜でも入手が容易で倫理上の問題もないので好ましい。

[0016] 酸処理に先立ち、羊膜に付着している細胞を除去し、除去しきれない細胞については死滅させることが好ましい。細胞の除去は、羊膜を緩衝液中で強く振盪することを繰り返すことにより行なうことができる。また、除去し切れなかった細胞を死滅させる処理としては、強アルカリ又はEDTA溶液による処理を挙げることができる。強アルカリとしては、水酸化ナトリウムのようなアルカリ金属水酸化物が好ましい。水酸化ナトリウムの場合、好ましい濃度は、特に限定されないが、通常、25mM～400mM、好ましくは50mM～200mM程度である。また、EDTA溶液中のEDTAの濃度は、通常、5mM～80mM、好ましくは10mM～40mM程度である。強アルカリ又はEDTAによる処理は、強アルカリ又はEDTAで羊膜を繰返し洗浄したり、又は羊膜を強アルカリ又はEDTA溶液中で強く振盪することを繰り返すことにより行なうことができる。このような処理を行

results of evaluation of the number of cells when cultured amniotic mesenchymal cells in various culture substrate

. Shows the evaluation results of the cell number when cultured human bone marrow-derived mesenchymal stem cells in a culture substrate that was coated was carried out in the embodiment of FIG 2 the present invention, human solubilized amnion composition of different concentrations is. Was performed in the embodiment of FIG 3] present invention, is a graph showing evaluation results of the cell number when cultured human bone marrow-derived mesenchymal stem cells in a culture substrate that is coated with human solubilized amnion composition. Was performed in the embodiment of FIG 4 the present invention, is a graph showing evaluation results of the number of cells when cultured human bone marrow-derived mesenchymal stem cells in various culture substrate. Is a graph showing the results in the embodiment of FIG 5] the present invention, the cultured human bone marrow-derived mesenchymal stem cells in various culture substrate, and compared the recovery of cultured cells collected by enzyme treatment. BEST MODE FOR CARRYING OUT the

invention

As

[0014] above, and are those obtained by the acid treatment under heating amniotic membrane, solubilized amnion composition of the present invention were included dissolution of amnion in a liquid medium is intended.

animal species

[0015] amnion is derived is not particularly limited as long as mammals, in the case of culturing cells to be administered or transplanted into a human or human cells, the safety and / or proliferation of cultured cells It is used as a raw material of human amniotic membrane from the point of view of gender is preferable. Is discharged along with the placenta after birth, the amniotic membrane, have been discarded as medical waste in humans amnion. Therefore, it is preferable since there is no ethical issues is readily available and also in human amniotic membrane.

be killed for cells to remove cells prior to

なった場合には、酸処理を行なう前に羊膜を水でよく洗浄して強アルカリやEDTAを洗浄除去することが好ましい。

[0017] 酸処理に用いる酸は、乾燥時に蒸発することにより、可溶化羊膜組成物を基体上にコーティング、乾燥して得られるコーティング材中に残留しない酸が好ましく、酢酸や塩化水素の水溶液が好ましい。酢酸水溶液の場合、酢酸濃度は特に限定されないが、通常、50mM～1M程度、好ましくは100mM～500mM程度である。

[0018] 酸処理に用いる酸の量（酸溶液の場合には酸溶液の量）は、羊膜を可溶化できる量であれば特に限定されないが、羊膜の湿重量１ｇ当り通常5mL～100mL、好ましくは10mL～50mLである。

[0019] 酸処理は、加熱下に行なわれる。酸処理時の温度は、特に限定されないが、通常、８０℃～１００℃であり、好ましくは８５℃～９５℃である。また、酸処理の時間は、羊膜が完全に溶けるまででよく、通常、６０分間～９０分間程度である。

[0020] 酸処理後は、塩基で処理することにより、組成物を中和することが好ましい。塩基は、可溶化羊膜組成物を基体上にコーティング、乾燥して得られるコーティング材中に残留しない塩基が好ましく、アンモニア水が好ましい。アンモニア水の濃度は、特に限定されないが、通常、25mM～400mM、好ましくは50mM～200mM程度である。中和後のpHは、中性、すなわち、pH6.5～7.5程度が好ましい。

[0021] 上記のようにして、可溶化された羊膜が媒体中に含まれる本発明の可溶化羊膜組成物が得られる。媒体は水が好ましく、上記の酸処理において、酸の水溶液を用いることにより、媒体が水である本発明の組成物を容易に得ることができる。あるいは、塩基の中和処理に、アンモニア水のような、塩基の水溶液を用いることにより、媒体が

[0016] acid treatment, is attached to the amniotic membrane, can not be removed is preferred. Removal of the cells can be carried out by repeating the shaking strongly buffer the amnion. Further, as a process that kills cells that can not be removed, it is possible to increase the treatment with EDTA solution or a strong alkali. The strong alkali, alkali metal hydroxides such as sodium hydroxide is preferred. For sodium hydroxide the preferred concentration is not particularly limited, typically, 25mM ~ 400mM, preferably about 50mM ~ 200mM. In addition, the concentration of EDTA in the EDTA solution is 10mM ~ 40mM usually about, 5mM ~ 80mM, preferably. Treatment with EDTA or a strong alkali can be carried out by or repeatedly washed amnion with EDTA or a strong alkali, or to repeat the shaking strongly EDTA solution or a strong alkali amnion. In the case of performing such processing, it is removed by washing EDTA or strong alkali and washed with water thoroughly amnion before performing the acid treatment.

by evaporation during drying, the acid does not remain in the coating material in the resulting coating, and dried on a substrate solubilized amnion composition is preferably acid used

the

[0017] The acid treatment, hydrogen chloride and ethyl aqueous solution is preferred. When aqueous acetic acid, the acetic acid concentration is not particularly limited, typically, 50mM ~ 1M about, preferably about 100mM ~ 500mM.

is not particularly limited as long as it is an amount that can be solubilized amnion but 5mL ~ usually per 1g wet weight of the amniotic membrane (the amount of the acid solution when the acid solution) the amount of acid used

the

[0018] Acid treatment 100mL, preferably 10mL ~ 50mL.

[0019] The acid treatment is carried out under heating. Temperature of the acid treatment is not particularly limited but is usually 100 ? 80 ? ~, is 95 ? 85 ? ~ preferably. In addition, well, usually, the acid treatment time is 60 minutes to about 90 minutes in the amnion until completely dissolved.

The

水である本発明の組成物を容易に得ることができる。

[0022] 本発明の可溶化羊膜組成物は、酢酸溶液の状態または凍結状態で保存することができる。

[0023] 本発明の可溶化羊膜組成物を基体上にコーティングし、乾燥することにより本発明の可溶化羊膜コーティング材を得ることができる。コーティングは、塗布、浸漬、スプレー等の周知の方法により行なうことができる。また、基体としては、通常の細胞培養に用いられている基体でよく、培養ディッシュやシャーレの底面やマイクロタイタープレートのウェルの底面でよい。基体の材質は、限定されるものではなく、通常、ガラスやポリスチレン等のプラスチックである。

[0024] 本発明の可溶化羊膜コーティング材は、細胞培養用基材として用いることができる。培養される細胞として何ら限定がないが、組成物が、ヒト由来の羊膜を可溶化した組成物から得られる場合には、ヒト細胞又はヒトに投与する細胞であることが細胞の増殖効率及び安全性の面から好ましい。本発明の細胞培養用基材上で培養される細胞は本実験では羊膜間葉細胞を用いたが、これに限定されるものではない。細胞の培養自体は、それぞれの細胞（幹細胞など）について知られている常法通りに行なうことができる。

[0025] 本発明にかかるコーティング材は、可溶化した羊膜の水溶液を基体に添加してコーティングを行なうので、羊膜成分を均一に塗布することが可能となり、従って、羊膜自体を用いた場合よりも基材がめくれ上がりにくいため長期の培養実験に適し、また、培養細胞が接着する培養ディッシュ上の位置による培養条件の影響を受けにくいという利点を有する。

[0026] 以下、本発明を実施例に基づきより具体的に説明する。もっとも、本発明は下記実施例に限定されるものではない。

実施例 1

[0020] After acid treatment, by treatment with a base, to neutralize the composition. The base, the base does not remain in the coating material in the resulting coating, and dried on a substrate solubilized amnion composition is preferably aqueous ammonia is preferred. The concentration of ammonia water is not particularly limited, typically, 25mM ~ 400mM, preferably about 50mM ~ 200mM. The pH after neutralization, neutral, ie, pH6.5 ~ 7.5 is preferably about.

As described

[0021] above, I can be obtained solubilized amnion composition of the present invention, amniotic membrane is solubilized is contained in the medium. Water is preferably the medium, the acid treatment described above, by using an aqueous solution of an acid, it is possible to easily obtain a composition of the present invention which is a water medium. Alternatively, by the neutralization of the base is used, such as ammonia water, an aqueous solution of a base, it is possible to easily obtain a composition of the present invention which is a water medium.

solubilized amnion composition

[0022] The present invention may be stored in a frozen state or acetic acid solution.

it is possible to obtain a solubilized amnion coating material of the present invention by coating on a substrate solubilized amnion composition

[0023] present invention, and dried. The coating can be performed coating, dipping, by methods known or spraying. Further, as the substrate, and may be a bottom surface of the well of a microtiter plate and the bottom surface of the petri dish and the culture dish at the base which is used in cell culture of normal. Is not limited, usually the material of the substrate is a plastic such as polystyrene or glass.

Solubilized amnion coating material

[0024] The present invention may be used as cell culture substrates. No way limited as a cell to be cultured, but may be obtained from a composition which composition is solubilized amnion of human origin, it is the cells that are administered to a human or human cells safely and proliferation efficiency of cells It is preferable in terms of sex. Cells cultured in the cell culture substrate of the

[0027] ヒト可溶化羊膜組成物の調製（その１） 次の方法により、ヒト羊膜の可溶化物を水中に含む組成物を調製した。 1) 分離羊膜 1.8 g （湿重量）を50 mlチューブに入れる。 2) 45 ml PBS (-) を加え、室温で５分間強く震盪。 3) 同上をさらに２回繰り返す。上清を捨てる。 4) 0.1 N NaOH 45 mlを加え、室温で強く震盪５分間。上清捨てる。 5) 0.1 N NaOH 45 mlを加え、室温で強く震盪５分間。上清捨てる。 6) 0.1 N NaOH 45 mlを加え、室温で強く一夜震盪。上清捨てる。 7) 0.1 N NaOH 45 mlを加え、室温で強く一夜震盪。上清捨てる。 8) 蒸留脱イオン水45 mlを加え、室温で強く震盪５分間。上清捨てる。 9) 同上をさらに２回繰り返す。上清を捨てる。 10) 45 mlの250 mM酢酸を加え、90℃湯煎で溶解するまで、時々震盪。 11) 0.1 Mアンモニア水で中和。 12)可溶化羊膜組成物として保存。

実施例 2

[0028] ヒト可溶化羊膜組成物の調製（その２） 次の方法により、ヒト羊膜の可溶化物を水中に含む組成物を調製した。 1) 分離羊膜 1.8 g （湿重量）を50 mlチューブに入れる。 2) 45 ml PBS (-) を加え、室温で５分間強く震盪。 3) 45 ml 0.1 NaOHで20 回洗浄。 4) 蒸留脱イオン水45 mlで３回洗浄。 5) 45 mlの250 mM酢酸を加え、90℃湯煎で溶解するまで、時々震盪。 6) 0.1 Mアンモニア水で中和。 7)可溶化羊膜組成物として保存。

実施例 3

[0029] ヒト可溶化羊膜組成物の調製（その３） 次の方法により、ヒト羊膜の可溶化物を水中に含む組成物を調製した。 1) 分離羊膜 1.8 g （湿重量）を50 mlチューブに入れる。 2) 45ml 0.02 M EDTAで7回洗浄。 3) 45ml PBSで 3回洗浄。 4) 蒸留脱イオン水45 mlで３回洗浄。 5) 45 mlの250 mM酢酸を加え、90℃湯煎で溶解するまで、時々震盪。 6) 0.1 Mアンモニア水で中和。 7) 蒸留脱イオン水で透析、3,000 RPM、3時間遠心。 8)可溶化羊膜組成物として保存。

present invention was used amniotic mesenchymal cells in this experiment, but not limited thereto. Culturing of the cells themselves, can be carried out in a conventional manner that is known each cell (such as stem cells).

It performs the coating can be added to a substrate solution of the amniotic membrane was solubilized, applying uniformly amnion components is possible, therefore, a coating material according

the

[0025] present invention using the amnion itself Further, suitable for long-term culture experiments for the substrate is less than curling up case, it has the advantage of not easily influenced by the culture conditions according to the position of the culture dish on cultured cells adhere.

[0026] below, I will explain more concretely by way of embodiments of the present invention. However, the present invention is not limited to the following examples.

Example 1

by the method of (1) The following preparation

[0027] person solubilized amnion composition, I was preparing a composition comprising in water solubilization of human amniotic membrane. I put in 50 ml of the tube weight) wet (1.8 g 1) separating the amniotic membrane. 2) 45 ml PBS (-) was added and shaken vigorously for 5 minutes at room temperature. I repeat two more times 3) Same as above. I discard the supernatant. Was added 4) 0.1 N NaOH 45 ml, strongly shaken for 5 minutes at room temperature. I discard the supernatant. Was added and 5) 0.1 N NaOH 45 ml, strongly shaken for 5 minutes at room temperature. I discard the supernatant. Addition of 6) 0.1 N NaOH 45 ml, strongly shaken overnight at room temperature. I discard the supernatant. Addition of 7) 0.1 N NaOH 45 ml, strongly shaken overnight at room temperature. I discard the supernatant. Was added to 45 ml 8) distilled deionized water, shaken vigorously for 5 minutes at room temperature. I discard the supernatant. I repeat two more times 9) Same as above. I discard the supernatant. Was added to 250 mM acetic acid 10) of 45 ml, until dissolved at 90 ? hot water, sometimes shaking. Neutralized with 11) 0.1

実施例 4

[0030] 培養基材としての利用（その１） ５０ micro lの実施例１で調製したヒト可溶化羊膜組成物（0.128mg/mL）を、培養面積0.32cm の培養ディッシュに入れ、培養ディッシュの底面を可溶化羊膜組成物でコーティングし、乾燥した。

[0031] 可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上に、間葉細胞であるヒト羊膜間葉細胞（約1,000個）を播種し、培養液として10 percent FBS添加したDMEM/F12を100 micro l入れ、３７℃で培養した。比較のため、I型コラーゲン、フィブロネクチン、ラミニン又はHEM（human extracellular matrix）をコーティングした市販の培養ディッシュ上でも同一条件下で培養した。さらに対照として、培養ディッシュ上に直接細胞を載せた場合及び培養ディッシュ上に蒸留脱イオン水100 micro lを入れて乾燥させて培養ディッシュ底面上に細胞を載せた場合についても試験した。培養開始３日目、６日目及び８日目に培養ディッシュの中に試薬（細胞増殖測定用試薬）を直接添加して、その吸光度を測定することにより、細胞数を評価した。

[0032] 比較に用いた各マトリックスでコーティングした培養ディッシュは以下のように調製した。（１）I型コラーゲンは、市販I型コラーゲン・コート９６ウェル・プレート（4860-010,Iwaki）を用いた。（２）フィブロネクチンは、市販のフィブロネクチン（Iwaki,02-070-002）を終濃度10 micro g/mLになるようリン酸緩衝液（PBS）で希釈した溶液を各50 micro lを各ウェルに分注して96ウェル培養ディッシュ（FALCON）の底面をコーティングし、37℃で一晩乾燥させ、その後PBSで2回洗浄して調製したものを用いた。（３）ラミニンは、市販のラミニン（Upstate,08-125）を終濃度34 micro g/mLになるようPBSで希釈した溶液を各50 micro lを各ウェルに分注して96ウェル培養ディッシュ（FALCON）の底面をコーティングし、37℃で一晩乾燥させ、その後PBSで2回洗浄して調製したものを用いた。（４）HEMは、

M aqueous ammonia. Save as 12) solubilized amnion composition.

Example 2

by the method (Part 2) The following preparation

[0028] Human solubilized amnion composition A composition was prepared containing the water solubilization of human amnion. I put in 50 ml of the tube weight) wet (1.8 g 1) separating the amniotic membrane. 2) 45 ml PBS (-) was added and shaken vigorously for 5 minutes at room temperature. Washing 20 times 3) 45 ml 0.1 NaOH. Washed three times with 45 ml 4) distilled deionized water. Was added to 250 mM acetic acid 5) of 45 ml, until dissolved at 90 ? hot water, sometimes shaking. Neutralized with 6) 0.1 M aqueous ammonia. Save as 7) solubilized amnion composition.

Example 3

by the method of (Part 3) The following preparation

[0029] person solubilized amnion composition, I was preparing a composition comprising in water solubilization of human amniotic membrane. I put in 50 ml of the tube weight) wet (1.8 g 1) separating the amniotic membrane. Washed seven times with 2) 45ml 0.02 M EDTA. Washed three times with 3) 45ml PBS. Washed three times with 45 ml 4) distilled deionized water. Was added to 250 mM acetic acid 5) of 45 ml, until dissolved at 90 ? hot water, sometimes shaking. Neutralized with 6) 0.1 M aqueous ammonia. Dialyzed against 7) distilled deionized water, centrifuged for 3 hours 3,000 RPM. Save as 8) solubilized amnion composition.

Example 4

was placed in a culture dish of culture area 0.32cm, human solubilized amnion composition prepared in Example 1 (Part 1) 50 micro l

used as a

[0030] culture substrate (0.128mg/mL), were coated with solubilized amnion composition the bottom of the culture dish, and dried.

the culture dish on a dry matter

市販のHEM（BD Biosciences,354237）を終濃度20 micro g/mLになるようPBSで希釈した溶液を各50 micro lを各ウエルに分注して96ウエル培養ディッシュ（FALCON）の底面をコーティングし、37℃で一晩乾燥させ、その後PBSで2回洗浄して調製したものを用いた。

[0033] 結果を図１に示す。図１より、本発明の可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上で間葉細胞の培養を行った場合、細胞数が最も多く、細胞増殖が最もよく促進されたことがわかる。

実施例 5

[0034] 培養基材としての利用（その２） それぞれ10 micro l、100 micro lのヒト可溶化羊膜組成物（0.128mg/mL）を蒸留水2mlに添加したヒト可溶化羊膜組成物水溶液を、培養面積9.6cm の培養ディッシュ（35-1008, FALCON）に入れ、培養ディッシュの底面を可溶化羊膜組成物でコーティングし、乾燥した。

[0035] 可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上に、組織幹細胞であるヒト骨髄由来間葉系幹細胞（hMSC: ells）（約50,000個）を載せ、培養液として10 percent FBS添加したDMEM/F12を100 micro l入れ、３７℃で培養した。比較対照として、培養ディッシュ上に直接細胞を載せた場合（NC: ontrol）及び培養ディッシュ上に蒸留脱イオン水100 micro lを入れて乾燥させて培養ディッシュ（DW: ater）底面上に細胞を載せた場合についても試験した。培養開始から３日目、６日目及び９日目に培養ディッシュの中に試薬（細胞増殖測定用試薬）を直接添加して、その吸光度を測定することにより、細胞数を評価した。また、培養開始から５日目、１０日目及び１５日目にも、同様の手法により細胞数を評価した。

[0036] 結果を図２及び３に示す。図２より、本発明の可溶化羊膜組成物の乾燥物がコーティングさ

[0031] solubilized amnion composition is coated, plated human amniotic mesenchymal cells, which are mesenchymal cells (about 1,000), and 10 percent FBS added as a culture was placed 100 micro l of DMEM/F12, and cultured at 37 ?. For comparison, were cultured under identical conditions but the culture dish on a commercially available coated HEM l collagen, fibronectin, laminin or a (human extracellular matrix). Were also tested for the case of carrying the cells into culture dishes bottom surface and dried by placing distilled deionized water 100 micro l to the culture dish and the case as a control, were placed on the cells directly in the culture dish further. By direct addition reagent (Cell Proliferation reagent for measuring) in the culture dish on day 3 culture initiation, on day 8 and day 6, and measuring the absorbance was evaluated cell number.

culture dishes that had been coated with the matrix used

in

[0032] comparison were prepared as follows. (1) l collagen was used (4860-010, Iwaki) commercial type I collagen-coated 96-well plate. (2) fibronectin, divided into each well of the 50 micro l solution was diluted with phosphate-buffered saline (PBS) to a final concentration of 10 micro g / mL fibronectin commercial (Iwaki,02-070-002) It was used to coat the bottom 96 well culture dish (FALCON) to refer, and dried overnight at 37 ?, was prepared by washing 2 times with PBS. The dispensed into each well of the 50 micro l (3) laminin, 96-well culture dishes (a solution diluted with PBS to a final concentration of 34 micro g / mL laminin commercially available (Upstate,08-125) It was used to coat the bottom of the FALCON), and dried overnight at 37 ?, was prepared by washing 2 times with PBS. The dispensed into each well of the 50 micro l (4) HEM and 96-well culture dish (FALCON solution was diluted in PBS to a final concentration of 20 micro g / mL HEM commercial (BD Biosciences, 354237) It was used to coat the bottom of), and dried overnight at 37 ?, was prepared by washing 2 times with PBS.

l is shown in Figure 1

the

[0033] results. From Figure 1, if it was cultured mesenchymal cells in culture dishes on the dry

れた培養ディッシュ上で培養を行った場合、該可溶化羊膜組成物の濃度依存的にhMSC細胞を増殖させることがわかる。

[0037] 図３より、本発明の可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上で培養を行った場合、hMSC細胞を有意に増殖させる効果が認められる。

実施例 6

[0038] 培養基材としての利用（その３） 実施例１で調製したヒト可溶化羊膜組成物５０ micro lを、培養面積0.32cm の培養ディッシュに入れ、96ウエル培養ディッシュ（FALCON）の底面を可溶化羊膜組成物でコーティングし、乾燥した。

[0039] 可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上に、hMSC（約1,000個）を播種し、培養液として10 percent FBS添加したDMEM/F12を100 micro l入れ、３７℃で培養した。比較のため、I型コラーゲン、フィブロネクチン、ラミニン又はHEM（human extracellular matrix）をコーティングした市販の培養ディッシュ上でも同一条件下で培養した。

[0040] 比較に用いた各マトリックスでコーティングした培養ディッシュは上記実施例４と同様の方法により調製した。

[0041] さらに対照として、培養ディッシュ上に直接細胞を載せた場合及び培養ディッシュ上に蒸留脱イオン水100 micro lを入れて乾燥させて培養ディッシュ底面上に細胞を載せた場合についても試験した。培養開始２日目、４日目及び８日目に培養ディッシュの中に試薬（細胞増殖測定用試薬）を直接添加して、その吸光度を測定することにより、細胞数を評価した。

[0042] 結果を図４に示す。図４より、本発明の可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上でhMSCの培養を行った場合、細

matter of the solubilized amnion composition of the present invention is coated, the number of cells the most, it can be seen that the cell proliferation was promoted best.

Example 5

solubilized human amniotic membrane composition was added to distilled water 2ml person solubilized amnion composition of, 100 micro l 10 micro l (2), respectively (0.128mg/mL)

use as a

[0034] culture substrate was placed (35-1008, FALCON) to the culture dish culture area 9.6cm, the aqueous solution was coated in soluble amnion composition to the bottom of the culture dish, and dried.

the culture dish on a dry matter

[0035] solubilized amnion composition is coated, human bone marrow-derived mesenchymal stem cells, which are tissue stem cells: is placed (hMSC ells) (about 50,000), as culture was placed 100 micro l of DMEM/F12 that was 10 percent FBS added, and incubated at 37 ?. Was placed on the cells: (ater DW) on the bottom surface culture dish and dried to put distilled deionized water 100 micro l in the culture dish and: (ontrol NC) If as a control, was placed the cell directly to the culture dish I was also tested for the case. By direct addition reagent (Cell Proliferation reagent for measuring) in the culture dish on day 3 from the start of culture, on day 9 and on day 6, and measuring the absorbance was evaluated cell number. Also, day 5 and day 15 and day 10, cells were assessed by the number of the same technique as the start of the culture.

l are shown in Figures 2 and 3

the

[0036] results. From Figure 2, when it was cultured in a culture dish drying of the solubilized amnion composition of the present invention is coated, it is understood that growing hMSC cells in a manner dependent on the concentration of solubilized amnion composition. from

[0037] FIG 3, when it was cultured in a culture dish drying of the solubilized amnion composition of the

胞数が最も多く、細胞増殖が最もよく促進されたことがわかる。

実施例 7

[0043] 間葉系幹細胞の接着率の検討 0.1mlのヒト可溶化羊膜組成物（0.128mg/mL）を、3.0cmペトリディッシュ（FALCON, 3001）に分注して底面を羊膜組成物でコーティングし、乾燥した。

[0044] 可溶化羊膜組成物の乾燥物がコーティングされた培養ディッシュ上に、hMSC（約2.0x10個）を播種し、培養液としてhMSC培養用の培養液であるMSCBM（正式名称：Mesenchymal Stem Cell Basal Medium）（カタログ番号：CAMBRE,入手先：Takara, PT-3001）を3.0 ml加えて、３７℃で培養した。比較のため、I型コラーゲンをコーティングした市販の100mm I型コラーゲン・コート・ディッシュ（Iwaki, 25020 COL1）、及び非コートディッシュ上でも同一条件下で培養した。培養開始から24時間以内に0.1 mlの0.1 %トリプシン・EDTAを添加し、37℃インキュベーター内でゆっくりと振盪させた。トリプシン・EDTA添加からそれぞれ1分、2分後に培地を添加することにより酵素反応を止めた。浮遊している細胞を回収し、細胞数を計測した。各系統について、同様の実験を３回繰り返し、浮遊細胞数の標準偏差を求めた。なお回収率は以下の式により計算した。 回収率（％）＝（回収した浮遊細胞数 x 100）÷（総細胞数）

[0045] 結果を図５に示す。可溶化羊膜コート・ディッシュ、コラーゲン・コート・ディッシュ、及び非コート・ディッシュで培養したhMSCの細胞回収率は、それぞれ55 plus/minus 6％，49 plus/minus 6％、及び35 plus/minus 8%であった。可溶化羊膜コート・ディッシュは、非コート・ディッシュと比較して、細胞が剥がれやすさに有意な差異が認められた。このことから、可溶化羊膜でコートした培養ディッシュを用いた場合には、短時間のトリプシン処理で高効率な細胞回収が可能となり、従って、トリプシンの酵素活性

present invention is coated, the effect of growing significantly hMSC cells is observed.

Example 6

was placed in a culture dish of culture area 0.32cm, a human solubilized amnion composition 50 micro l as prepared in (3) in Example 1 utilizing

as

[0038] the culture substrate, 96-well culture dish (FALCON) were coated with solubilized amnion composition of the bottom surface, and dried.

the culture dish on a dry matter

[0039] solubilized amnion composition is coated, seeded hMSC (about 1,000), were placed 100 micro l of DMEM/F12 that was 10 percent FBS added as a culture medium, l and incubated at 37 ?. For comparison, were cultured under identical conditions but the culture dish on a commercially available coated HEM I collagen, fibronectin, laminin or a (human extracellular matrix).

was prepared by a method similar culture dishes coated with the matrix used in

[0040] compared to that of Example 4.

even when it is loaded with cells in the culture dish bottom surface and dried by placing distilled deionized water 100 micro l to the culture dish and the case as a control, were placed on the cells directly in the culture dish further

[0041]

were tested. By direct addition reagent (Cell Proliferation reagent for measuring) in the culture dish on day 2 the initiation of culture, the days 4 and 8, and its absorbance measured and evaluated cell number.

l is shown in Figure 4

the

[0042] results. From Figure 4, when it was cultured in hMSC culture dish drying of the solubilized amnion composition of the present invention is coated, the number of cells the most, it can be seen that the cell proliferation was promoted best.

Example 7

による細胞へのダメージを軽減できるという優れた技術的効果を奏するといえる。

[0046] 一方、図５に示す結果から、可溶化羊膜コート・ディッシュとコラーゲン・コート・ディッシュを用いた場合で、細胞回収率に有意な差異は認められない。しかしながら、コラーゲンは、その精製過程において種々の酵素などを使用しており、精製過程で使用した物質の残留が問題となるために、コラーゲン存在下の条件で培養した細胞をそのまま体内に移植することは現段階では危険を伴う。その一方で、可溶化羊膜コート・ディッシュであれば、羊膜を可溶化する際に使用する酢酸及びアンモニアは揮発することで完全に除去することが可能であり、可溶化羊膜コートした培養ディッシュ上で培養した細胞はそのまま細胞移植に用いたとしても精製過程で使用する物質の残留に伴う危険は回避できるという優れた技術的特徴を有する。

実施例 8

[0047] 可溶化羊膜組成物の組成 実施例１で調製した可溶化羊膜組成物の組成を分析した。その結果、主な成分は次の通りであった。

[0048] １． 蛋白質濃度： 60~300 micro g / ml
２． 脂質分析：燐脂質、糖脂質、ガングリオシドを含有している ３． コラーゲン：含有を確認
４． ラミニン：可溶化するまえの羊膜に存在を確認（免疫染色）

[0049] 以上の結果、本発明の羊膜可溶化組成物は、コラーゲンのみではなく、生体内の膜成分に近い組成をもっていることが判明した。

The amniotic membrane composition and the bottom is dispensed 3.0cm Petri dish (FALCON, 3001), solubilized human amniotic membrane composition of study 0.1ml of adhesion rate

[0043] mesenchymal stem cells (0.128mg/mL) were coated with things, and then dried.

the culture dish on a dry matter

[0044] solubilized amnion composition is coated, seeded hMSC (about 2.0x10 pieces), MSCBM is a culture of hMSC culture as culture (official name: Mesenchymal Stem Cell Basal Medium) (catalog number: CAMBRE, Where to get: In addition 3.0 ml Takara, the PT-3001), were cultured at 37 ?. For comparison, I was cultured under the same conditions, even on non-coated dishes and, (, 25020 COL1 Iwaki) 100mm I collagen Court commercially available dish coated with type I collagen. Was added 0.1 percent trypsin • EDTA in 0.1 ml of 24 hours from the start of the culture, were gently shaken at 37 ? incubator. 1 min each of trypsin • EDTA was added and the enzyme reaction stopped by addition of medium 2 minutes. The harvested cells are suspended, and the number of cells was counted. For each strain, was repeated three times and similar experiments, to determine the standard deviation of the floating cell number. The I was calculated by the following equation recovery. The (floating cell number was recovered x100) • recovery rate (percent) = (total cell number)

I is shown in Figure 5

the

[0045] results. And cell recovery rate of hMSC cultured in non-coated dish solubilized amnion Court dish, and collagen-coated dish, is, 55 plus / minus 6 percent, 49 plus / minus 6 percent, 35 plus / minus 8 percent, respectively were. In comparison with non-coated dish, solubilized amnion coat dish was observed a significant difference in the ease of peeling cells. Therefore, in the case of using a culture dish coated with solubilized amnion cells recovered with high efficiency becomes possible with brief trypsinization and therefore excellent ability to reduce damage to the cells by the enzymatic activity of trypsin It can be said that it exhibits the technical effect was.

Meanwhile

[0046] From the results shown in FIG 5, in the case of using collagen-coated dishes and solubilized amnion coated dish, significant difference was shown on cell recovery. However, collagen, which uses such as various enzymes in the purification process, that for the remaining materials used in the purification process is a problem, it is implanted in the body as the cells cultured under the conditions of collagen presence I potentially dangerous at this stage. On the other hand, if the solubilized amnion coated dish, it is possible to remove completely by ammonia and acetic acid is used to solubilize the amnion volatilized, a culture dish, which was solubilized amnion coated Cells were cultured with the technical features outstanding ability to avoid the hazards involved residual substances used in the purification process as was used for cell transplantation as it is.

Example 8

we analyzed the composition of the solubilized amnion composition was prepared with the composition in Example 1

[0047] solubilized amnion composition. As a result, the main components were as follows.

[0048] 1. Protein concentration: 60 ~ 300 micro g / ml 2. 3, which contains phospholipids, glycolipids, gangliosides: lipid analysis. Check 4 containing: collagen. The laminin: confirm the presence in the amniotic membranes prior to solubilization (immunostaining)

Results

[0049] above, the amniotic membrane solubilized composition of the present invention, it is possible not only collagen, it has a composition close to the membrane components in vivo has been found.

Citing: JP2001161353 A2 ; WO04076631 A2 ;

Cited by: JP2016504932 T2 ;

Non patent citations: KOBAYASHI M. ET AL.: 'Enhancement of mesenchymal cell proliferation by using solubilized amniotic membrane as scaffold' HUM. CELL vol. 21, no. 1, 2008, page A5 + ABSTR. NO. O-02

KOBAYASHI M. ET AL.: 'Enhancement of mesenchymal cell proliferation by using solubilized amniotic membrane as scaffold' HUM. CELL vol. 21, no. 1,

2008, page A5 + ABSTR. NO. O-02, XP008123513

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KUMAR T.R ET AL.: 'Biocompatible collagen scaffolds from a human amniotic membrane: physicochemical and in vitro culture characteristics' J. BIOMATER. SCI. POLYMER EDN. vol. 14, no. 7, 2003, pages 689 - 706

KUMAR T.R ET AL.: 'Biocompatible collagen scaffolds from a human amniotic membrane: physicochemical and in vitro culture characteristics' J. BIOMATER. SCI. POLYMER EDN. vol. 14, no. 7, 2003, pages 689 - 706, XP009117131

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MORIO UENO ET AL. PNAS vol. 103, no. 25, 20 June 2006, pages 9554 - 9559

See also references of EP 2149601A1

See also references of EP 2149601A1

T. R. KUMAR ET AL. J. BIOMATER. SCI. POLYMER EDN. vol. 14, no. 7, 2003, pages 689 - 706

Electronic Acknowledgement Receipt

EFS ID:	32244994
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Kevin MacDonald/Chelsie Bailey
Filer Authorized By:	Kevin MacDonald
Attorney Docket Number:	S1860.70000US01
Receipt Date:	04-APR-2018
Filing Date:	08-JUL-2015
Time Stamp:	14:52:55
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	S186070000US01-TRN-KZM.pdf	25018 9aee6edf3df5f09a38a8c75457038cc6c089d979	no	1
Warnings:					

Information:					
2		S186070000US01-IDS-KZM.pdf	51726 23ef90fbab12e03e551d5e788c47dab98cd5accc	yes	4
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Information Disclosure Statement (IDS) Form (SB08)		4	4	
	Transmittal Letter		1	3	
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Information:					
3	Non Patent Literature	Satoh_Zoolog_Sci_2012_v29_p191.pdf	1444223 69a0b4bc42c2912ebd9e8bd3f13ca08116b367f4	no	7
Warnings:					
Information:					
4	Foreign Reference	WO08129997_Eng_Trans_QC.pdf	7462063 831b1a84adb93241e9eb6d764d87f52798b1099b	no	17
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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	R. A. Davis
Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

Form PTO-1449

Copies of cited references

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Kevin MacDonald/		
Printed name	Kevin MacDonald, PhD		
Date	April 4, 2018	Reg. No.	74,522

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: April 4, 2018 Electronic Signature for Chelsie Bailey: /Chelsie Bailey/

CONSIDERED: /R.A.D/
04/12/2018

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, : Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian : Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; 371 date: Jul. 8, 2015 : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

EXPERT DECLARATION UNDER 37 C.F.R. 1.132
TO ACCOMPANY THE FILING OF A REQUEST FOR CONTINUED EXAMINATION IN THE SUBJECT APPLICATION

THE COMMISSIONER OF PATENTS & EXAMINER DAVIS:

1. I hereby declare that I am the same Ashley W. Seifert whose *Curriculum Vitae* is attached to this Declaration, and that I am the same Ashley W. Seifert named as first author in the 2012 publication "Skin Regeneration in Adult Axolotls: A Blueprint for Scar-Free Healing in Vertebrates", (citable as PLoS ONE 7(4): e32875. doi:10.1371/journal.pone.0032875), which has been cited on the record by the Patent Examiner to support rejection of the claims pending in the subject application as lacking novelty or, in combination with other references, as being unpatentable for obviousness. I hereby further note that I am retained as a consultant by a licensee of rights in the subject application.

2. I am aware that the Examiner, in an Office Action dated 10/18/2017, has rejected certain claims as lacking novelty, in light of Satoh et a1. (2012). I am also aware of, have read, and I concur with the arguments and claim amendments that are being filed concurrently with this Declaration, in support of the novelty of the pending claims, notwithstanding citation of the Satoh reference. Furthermore, I have read and concur with the previously submitted Declaration of Dr. Ryanne Early, the inventor in the subject application, filed to support the patentability of the pending claims. The ECM_{iv} and ECM_{ev} nomenclature provided in that Declaration is adopted and

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referred to herein, along with an additional term defined herein, namely "ECM_{evxx}", used to refer to a cross-linked ECM_{ev} product produced according to the Satoh reference, as discussed further herein below.

3. It is my opinion that the product disclosed and claimed in the subject application (referred to herein as ECM_{ev}) is a necessarily different product (physicochemically and biochemically, exhibiting different surface chemistry interaction potential and functionality) than the glutaraldehyde cross-linked product ("ECM_{evxx}") the production of which is disclosed in the Satoh reference.

4. FIG. 5 in the subject application provides a histological evaluation of H&E-stained, paired native and post-processed sections of axolotl skin tissue. Clearly, even from just a gross morphological perspective, ECM_{iv} and ECM_{ev} are quite different, if for no other reason than that, in the ECM_{ev}, the cellular constituents present in ECM_{iv} have been removed. I understand that the Patent Examiner has recognized this and many other previously identified distinctions between ECM_{iv} and ECM_{ev}, by withdrawing a previously asserted rejection based on 35 U.S.C. section 101; thereby recognizing that the ECM_{ev} claimed in this patent application is not the same material or product as ECM_{iv} albeit retaining the natural architecture thereof.

5. In executing this Declaration, I am providing my opinion, as an expert in the field of tissue regeneration, (and the underlying and related fields, to the extent practically necessary, of, for example, biochemistry, tissue histological examination, microscopy, chemistry, and biology), that the Satoh product, created after removal of a sample of ECM_{iv} from its *in vivo* environment and subsequently processed, including via glutaraldehyde fixation, results in a product, ECM_{evxx}, which differs in a number of significant ways, as further attested to below, from ECM_{ev} and from ECM_{iv}, just as ECM_{ev} differs in a number of significant ways from ECM_{iv}. (please see the Table below in section 8).

6. As noted in Paragraph 4 herein, without more, simply the irrefutable basis that the Satoh product has been produced by a process that includes fixation, thereby producing ECM_{evxx}, it is my opinion that it is excluded from the claimed subject matter, it is different from the claimed

subject matter, and Satoh does not teach or suggest the subject matter taught and claimed in the subject application. What is claimed excludes, by its express terms, a tissue sample that has undergone fixation by treatment with glutaraldehyde[1], formaldehyde, (aka formalin), or combinations thereof, in that the present invention as claimed requires:

"obtaining a tissue sample from a urodele [i.e. axolotl, thus far, as in Satoh, the same ECM_{iv} starting material) where the tissue sample retains the **extracellular matrix** (ECM) which has been decellularized **by a process which preserves the structural and functional integrity of the ECM that it had in the ECM_{iv} tissue sample**." This is certainly not the case for the Satoh material if for no other reason than it has undergone glutaraldehyde fixation. While cells may have been disrupted in the course of production of Satoh's ECM_{evxx} product, in that product, pursuant to its method of production, disrupted cellular components can be retained in the cross-linked ECM as a result of glutaraldehyde treatment of the ECM in the presence of cellular debris.

7. I further opine that the intended use of the acellular material produced according to Satoh is revealed on page 194 of Satoh, right hand column, which provides commentary on Figure 6 in Satoh. Essentially, what Satoh reports is that the glutaraldehyde fixed, acellular collagen sheet of ECM_{evxx}, when placed in an environment otherwise conducive for accessory limb regeneration to be observed, (nerve rerouting or deviation + skin wounding), **the presence of the ECM_{evxx} prevents blastema formation and limb regeneration in the axolotl species from which the tissue was obtained.** Thus, quite the contrary to finding a demonstration in Satoh that an ECM_{ev} product could be produced with sufficiently depleted cellular components to make the product suitable for use as a xenograft, Satoh actually shows that in the axolotl, the ECM_{evxx} product prevents regeneration. This in no way can stand as a showing that the ECM_{evxx} is the same as

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[1] See, for example, https://en.wikipedia.org/wiki/Glutaraldehyde, which provides: "Glutaraldehyde is used in biochemistry applications as an amine-reactive homobifunctional **crosslinker** and **fixative** prior to SDS-PAGE, staining, or electron microscopy. **It kills cells quickly** by **crosslinking their proteins** and is usually employed alone or mixed **with formaldehyde** as the first of two fixative processes to stabilize specimens such as bacteria, plant material, and human cells. A second fixative procedure uses osmium tetroxide to crosslink and stabilize cell and organelle membrane lipids. Fixation is usually followed by dehydration of the tissue in ethanol or acetone, followed by embedding in an epoxy resin or acrylic resin. Another application for treatment of proteins with glutaraldehyde is the inactivation of bacterial toxins to create toxoid vaccines, e.g., the pertussis (whooping cough) toxoid component in the Boostrix Tdap vaccine produced by GlaxoSmithKline. In a related application, glutaraldehyde is sometimes employed in the tanning of leather and in embalming."

ECM_{ev} according to the present invention, nor would it inspire one skilled in the art to explore use of axolotl tissue for the purpose of making a tissue product suitable for use as a xenograft.

8. It is my opinion that the following table makes it clear that ECM_{evxx} is at least as dissimilar (non-identical) to either ECM_{iv} or ECM_{ev}, as ECM_{ev} is dissimilar (non-identical) to ECM_{iv}, particularly when considering the known presence or absence of particular cellular components, and the known function/intended-use of the product:

	ECM_{iv}	**ECM_{ev}** (Present invention)	**ECM_{evxx}** (Satoh)
	Present (P)/Absent (A) (function or intended use)	Present (P)/Absent (A) (function or intended use)	Present (P)/Absent (A) (function or intended use)
Intact Cells	P, (natural architecture with cells present)	A, (natural architecture retained due to absence of cross-linking; cells and cellular debris removed)	A, (non-natural architecture due to cross-linking; cells destroyed but cellular debris present due to cross-linking)
DNA	P (contained within intact cells)	A (i.e. while the natural architecture is retained, cellular material has been removed, as evidenced by the reduced A260/A280 of ECM_{ev} as compared to ECM_{iv})	A (presumably DNase treatment has removed DNA; no mention is made of RNase and RNA or any other cellular components or debris retained by cross-linking)
Cross-linking	A	A	P
Function/intended-use of product	*In Vivo* ECM_{iv} is a primarily collagenous matrix which provides structural support and is laid down by and between cells as they grow adjacent each other.	ECM_{ev} per the present invention is ECM*iv* which, while maintaining the natural architecture of ECM*iv* (by avoiding the use of cross-linking agents which lock the naturally variable and flexible morphology of ECM_{iv} into cross-link constrained morphologies and configurations), has been debrided of cellular material to reduce antigenicity to make the material suitable for use as a xenograft.	ECM_{evxx}, per Satoh is ECM*iv* which has been treated by fixation, with cross-linking agents which lock the naturally variable and flexible morphology of ECM_{iv} into cross-link constrained morphologies and configurations, and which has been treated in such a way that although intact cells have been destroyed, it is evident from Satoh itself that nuclei have been retained and require DNase treatment to remove that material. That is, the intended use of the Satoh product was to interfere with natural interactions at the injury site to inhibit regeneration, which is contrary to the intended use of the claimed product.

9. As previously stated in the Declaration of inventor Ryanne Early, what is clear from the data presented in Figure 5 in the present patent disclosure is that ECM_{iv} is an intricate series of

structures, layers, and conduits, tightly interlaced with cells, (which contain DNA), cell components, proteins, and the like. ECM_{iv}, replete as it is with cells and structure accommodating those cells, is not **"the same"** as ECM_{ev} following decellularization and removal of cellular debris, according to the process of production taught and claimed in the subject patent disclosure. Per this disclosure, ECM as it exists *in vivo*, i.e. ECM_{iv}, is isolated, nucleic acids are removed as a marker of removal of cells, and cellular debris is removed, to produce Urodele connective tissue, (also referred to herein as ECM_{ev}), characterized by retention of structural and functional integrity coupled with reduced or eliminated antigenicity, due to removal of sufficient cellular components to achieve that characteristic, making it suitable for use as a xenograft. Nothing like this is disclosed, taught or suggested in the Satoh reference, which is cross-linked to itself and to cellular debris. That is, even if it were contended that Satoh "attempts" to create something similar to what is disclosed and claimed in the present application, the Satoh processing is different in that fixation is used. Thus, to the extent that Satoh discloses or suggests production of an acellular graft, Satoh does so through different process steps, which results in production of a different product, for a different purpose.

10. It is my opinion that Satoh does not teach an ECM_{ev} product that is the same or even similar to the presently claimed ECM_{ev}. This opinion is based, at least in part, on the disclosure in the Satoh reference, at page 192, bottom of left hand column to the top of the right-hand column, where Satoh provides the following description of how an "acellular collagen sheet" was produced from axolotl tissue:

"The acellular collagen sheet that we used was prepared as follows: skin was isolated from the upper limb and incubated in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours, **and then incubated in water with 0.01% Tween 20[2], PMSF (1.74 μg/ml), and glutaraldehyde** (20.18 μg/ml) for two days, resulting in an acellular [3]ECM sheet. **The remaining**

[2] While this Declaration and the prior Declaration of Dr. Ryanne Early, and the remarks and arguments that have been made on the record have focused primarily on Satoh's teaching of the use of glutaraldehyde in the course of preparing the ECM_{evxx} product, it should not be ignored that Satoh also teaches the use of Tween 20, a detergent, and PMSF, a protease inhibitor

[3] Perhaps "acellular" in the sense of no remaining intact cells, but certainly not free from cellular debris - as is clear from the very next sentence, addressing the "remaining nuclei" which are removed by extensive DNase treatment. There would be no need to remove this material at this stage in the Satoh process had those materials not been trapped by the fixation process employed by Satoh. Inherently, in the process for manufacture of the ECM_{ev} product of the present invention, which makes no mention of use of

nuclei were removed by incubating the sheet in DNase solution for 1 h at 3°C (Promega). The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS **and placed on the rerouted nerve."**

In my opinion, the Satoh process, and the product made by that process, may properly be defined as:

Step a:

Isolate skin from the upper limb of an axolotl and incubate the skin sample in phosphate-buffered saline (PBS) containing 0.05% EDTA for several hours; the EDTA being present to sequester metal ions; intact cells present in the ECM_{iv} sample may or may not rupture during this step; if the cells rupture, the cellular components, such as nucleic acids, (see below) are not removed.

Step b:

Incubate the material from Step a in water with 0.01% Tween 20[4], PMSF (1.74 μg/ml), and glutaraldehyde (20.18 μg/ml) for two days, resulting in an acellular [5] ECM sheet.

Step c:

The remaining nuclei were removed by incubating the sheet in DNase solution for 1 h at 3°C (Promega).[6]

Step d:

[4] While this Declaration and the prior Declaration of Dr. Ryanne Early, and the remarks and arguments that have been made on the record have focused primarily on Satoh's teaching of the use of glutaraldehyde in the course of preparing the ECM_{evxx} product, it should not be ignored that Satoh also teaches the use of Tween 20, a detergent, and PMSF, a protease inhibitor, the use of either or both of which will have additional effects on the ECM_{evxx} of Satoh as compared to the ECM_{ev} of the present invention.

[5] Perhaps "acellular" in the sense of no remaining intact cells, but certainly not free from cellular debris - as is clear from the very next sentence, addressing the "remaining nuclei" which are removed by extensive DNase treatment. There would be no need to remove this material at this stage in the Satoh process had the nuclei, and presumably other cellular components, including other organelles, proteins, etc. not been trapped by the fixation process employed by Satoh. Inherently, in the process for manufacture of the ECM_{ev} product of the present invention, the effect of these other agents we be additive to create additional differences between the Satoh product and the product of the subject invention, beyond the effect of just the cross-linking that occurs in step (b) due to the presence of glutaraldehyde.

[6] As noted above, there would be no need to remove these nuclei had they not been trapped by the cross-linking step (b).

The sheet was fluorescently stained with FITC-I (Dojindo) according to the manufacturer's protocol. The sheet was washed with PBS and placed *in vivo* on top of the rerouted nerve. While FITC staining is not necessarily specifically excluded in the process of manufacture of the product of the subject invention, it is certainly not required in that process. It is included in the Satoh process so that the inclusion of green fluorescent dye could be used to demonstrate placement of the ECM_{evxx} and that the ECM_{evxx} prevented interaction of the deviated nerve and the epidermis when introduced into the Satoh model. The product produced by this process is a cross-linked tissue in which the cells have been disrupted, but the debris from the disrupted cells is not necessarily removed, and which is FITC labeled.

11. Satoh also only discloses the production of a miniscule amount (due to the tiny amount of skin available from the axolotl upper limb) of "an acellular ECM sheet". Furthermore, the Satoh material is obtained from an axolotl in the form of a cross-linked matrix (due to the glutaraldehyde) which would fix and retain, in a given confirmation, the structure of ECM, while altering functional integrity of the tissue by changing access to active sites on the ECM itself. Glutaraldehyde cross-linking results in production of a much stiffer ECM material with non-native chemistry, physical properties, antigenic/immunogenic profile, and resorption/remodeling profile. In addition, by cross-linking the material produced according to Satoh, both the cross linking itself and the fact that such cross linking would covalently bind any cellular debris still present (since Satoh teaches treatment of the entire native ECM with detergent to rupture cells without teaching any separation of such material prior to glutaraldehyde treatment), the Satoh material fails to meet the requirement in the present claims that sufficient cellular components are removed to reduce or eliminate antigenicity of the biomaterial as a xenograft. Per Satoh, this cannot be achieved due to the covalent cross-linking of cellular debris to the connective tissue matrix.

12. By contrast, the product according to the present invention is produced by a process comprising decellularizing of the sample to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to render it suitable for use as a xenograft while retaining structural and functional integrity of the natural, cellular, tissue. Accordingly, the product and process of producing the product disclosed in Satoh is neither the same as or even

similar to the product and process of producing the product pursuant to the claims pending in the subject application.

13. I am aware that Claims 5, 20-22, 26, 36-38, and 40-61, stand rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012); that Asem is being cited as providing a teaching of decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40); that the Asem ECM is said to be in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64); that the ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). I further understand that the Examiner has conceded that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, that notwithstanding, I further understand that the Examiner is asserting that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious."

14. It is my opinion, as an expert in this field, and as principal author in the Seifert publication which the Examiner is citing, in combination with the Asem reference, that the combination proposed by the Examiner in rejecting the claims is not defensible. For the additional reasons that follow, it is my opinion that the Seifert et al. reference, if anything, teaches away from the combination with Asem proposed by the Examiner, in particular because the Seifert reference (page 15, bottom left column under the heading "ECM Production and New Tissue Formation"),

reports that "...the slow deposition of new extracellular matrix (ECM) and its unique molecular composition suggests newly synthesized axolotl ECM may antagonize fibrosis and promote regeneration (Figure 11)". Furthermore, (see page 16, left column, bottom before next heading), where it is stated in Seifert that: "These data suggest that the composition of ECM produced during a regenerative response may have an anti-fibrotic effect that helps to recapitulate embryonic development and promote a regenerative response". Thus, in fact, one of ordinary skill in the art encountering Seifert, while being apprised of Asem might understand that it would be worth trying to see if components of regenerating ECM, rather than intact, uninjured ECM, as disclosed and claimed in the subject application, may be useful as a tissue graft. Success in achieving this result is not disclosed in Seifert alone, in Asem alone, or in any combination of the two references. Furthermore, in my opinion, there is no basis provided in the Seifert publication to support the proposition that the Seifert reference provides "evidence that it would have been obvious to use Urodele or axolotl as a functionally equivalent amphibian ECM with a reasonable expectation for successfully obtaining a functional decellularized ECM." The Examiner has not provided any citation to any aspect of the Seifert publication which would support this allegation.

15. The Examiner asserts that it would have been obvious to use "any known functionally equivalent ECM" of the Asem follicular ECM as a substitute for the Asem follicular ECM. There is no teaching in Asem (or in Seifert et al.) that there is any tissue (other than placental tissue) that is the "functional equivalent" to follicular ECM. Asem teaches, if anything, that follicular tissue, like placental tissue, is unique, and the processes for making the product in Asem, as in Seifert, as in Satoh, as indeed in the present application, is *sui generis*. The Asem reference is further distinguished as being directed to basement membrane, which is not ECM *per se,* but only one constituent of ECM. Furthermore, Asem sources their basement membrane from a unique source, the ovarian follicle, which is essentially a thin basement membrane surrounding an oocyte (egg). In contrast, the ECMev according to the present invention is derived from mature skin, which is completely different in structure, form and function than the Asem basement membrane material, and was selected not for the basement membrane, but for the dermal tissue in combination with the basement membrane.

16. Follicular ECM is found in small quantities and is relatively difficult to obtain. Asem teaches that follicular basement membrane tissue is collected from eggs, including eggs of reptiles. The basement membrane ECM is harvested from such tissue. Asem asserts that follicular tissue is more easily obtained, however, than placental tissue. There is no basis to suggest that ECM tissue sources other than follicular tissue would be the functional equivalent of basement membrane ECM from follicular tissue. One of ordinary skill in the art would not go to the trouble of collecting small quantities of follicular tissue from eggs if any source of ECM could be readily substituted for follicular ECM. Furthermore, even assuming Asem were read as leading one skilled in the art to consider obtaining follicular ECM from a Urodele or even from an axolotl - Seifert does not teach production of the ECM produced according to the present invention; neither does Satoh; neither does Asem; neither do any or all of them combined do so. Thus, ECM produced according to Asem, even if made from Urodele or even axolotl basement membrane follicular tissue, would be a different product than if that same follicular tissue were obtained from Urodele or axolotl follicular tissue and treated according to the process of the present claims. This is made plain from Figure 1 of the Seifert reference, annotated to show tissues which are included in ECM_{ev} following decellularization to remove cells and debris form the dermis and basement membrane. The basement membrane is a tissue structure itself, but also a component of any epithelial tissue. The basement membrane is a component of the epidermis, intestine, etc., whereas ECM is constituted by a large class of proteins which primarily exist outside cells. For instance, the human MATRISOME, comprises over 200 proteins (http://web.mit.edu/hyneslab/matrisome/). Accordingly, in my opinion, citing the Asem reference, which is focused on use of basement membrane (comprised of just a few types of proteins) and asserting that such a structure is the same as or anticipates a product comprising ECMev, is equivalent to asserting that a simple protein formed from just a few types of amino acids (e.g. albumin), because it is formed from amino acids, is equivalent to, or is the same as or anticipates all the proteins on earth, which is not the case even though all proteins share some basic amino acids.

17. Seifert does not suggest anything to the contrary. In fact, Seifert teaches that one would want to avoid the presence of native ECM in promoting scar-free healing, teaching away from the combination suggested by the Examiner. For these reasons, it is my opinion that the cited

Satoh, Asem, and Seifert references, considered alone or in combination, do not teach or suggest a native (uninjured), sterile, isolated, decellularized Urodele extracellular matrix (ECM) produced by a process comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial thereby making it suitable for use as a xenograft."

18. While Seifert mentions ECM and extracellular matrix throughout, this is all in the context of *in vivo* ECM production as it relates to regeneration or scar tissue formation. In fact, a thorough review of Seifert has failed to reveal any disclosure of production of anything like an ECM_{ev} acquired as native ECM. The closest that has been found to any such disclosure in Seifert is found on page 17 which provides:

"For histological and immunohistochemical analysis, **harvested tissues were fixed in 10% NBF** at 4°C for 16–24hr, washed in PBS and dehydrated to 70% EtOH. Samples were stored at 4°C and **processed for paraffin embedding**. Samples were cut at 5μm. For frozen sections, samples were fixed for 1hr in 10%NBF at 4°C, washed in PBS, equilibrated in sucrose and OCT freezing medium and frozen in OCT."

NBF is Neutral Buffered Formalin, or formaldehyde, a cross-linking agent. Accordingly, much like the *ex vivo* tissue produced by Satoh, the Seifert material, whether considered alone or in combination with the Asem publication, fails to teach or suggest a decellularized ECM product with reduced antigenicity.

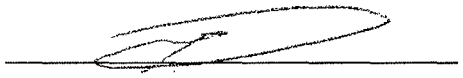
19. It is my opinion that it cannot validly be stated that Seifert's histological section preparation steps, carried out to produce tissue samples adapted for analysis of the scar free tissue regeneration process that occurs *in vivo* in the axolotl, which includes formalin fixing axolotl tissue specimens at different stages of *in vivo* wound healing, is adequate to teach or suggests a process used to prepare a tissue product, or the tissue product produced thereby, which is not formalin fixed, is decellularized, and thereby made suitable for implantation into another species than that from which it was derived.

20. In my opinion, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of retaining structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft.

21. It is my opinion that the combination proposed by the Examiner in rejecting the claims is not based on sound reasoning and is made only with hindsight, after study of the present application and claims. The Examiner has conceded that Asem does not teach the ECM wherein the amphibian is a Urodele or axolotl. To address this deficit in Asem, the Examiner combines the Asem teaching with that of Seifert. However, the only mention of ECM in Seifert is in the context of ECM produced *in vivo* in response to injury and its role in scar-free tissue regeneration within that species. Further, as noted above, it is simply illogical to assert that Seifert's teaching of histological section preparation steps for fixing and embedding a tissue specimen and the product produced by such a method anticipates, teaches or suggests a process to prepare a tissue product and the product produced which is suitable for implantation as a xenograft. Seifert neither teaches nor suggests the present process or product. Those skilled in the art aware of Asem's teaching, had they turned to or become aware of the Seifert reference, at most, may have thereby considered the possibility of acquiring ECM produced in response to injury, rather than, as in the subject application, using native, uninjured ECM_{ev}, which does not contain the same components as the ECM produced during healing *in vivo* as defined in detail in the Seifert publication.

22. For all of the foregoing reasons, it is my opinion that the references cited by the Examiner do not teach or suggest the claimed invention, alone or in combination, that is a decellularized Urodele extracellular matrix (ECM) produced by a process comprising:
(A) obtaining a tissue sample from a Urodele, which tissue sample comprises extracellular matrix, cells and ground substance, and
(B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft.

The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.



02 - 15 - 2018

Ashley W. Seifert, Ph.D. Date

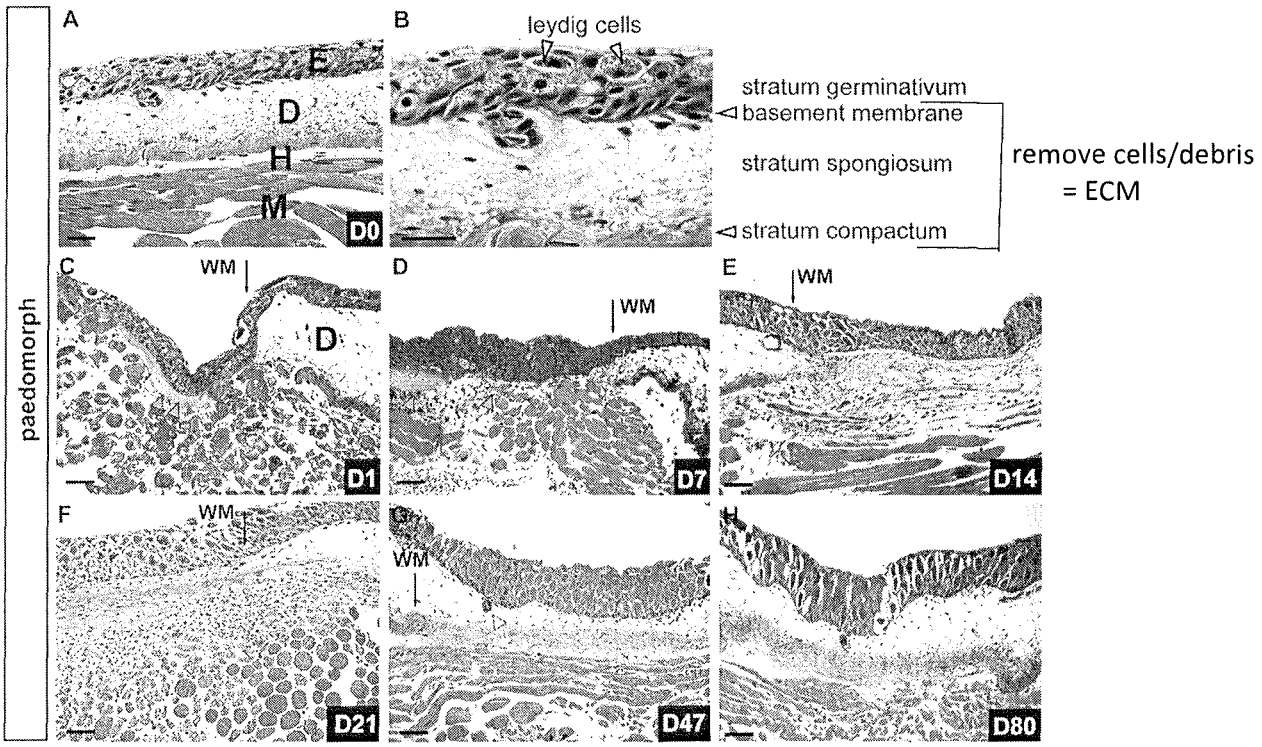


Figure 1. Scar-free healing of full thickness excisional wounds in adult axolotls. A) Masson's trichrome staining of uninjured dorsal flank skin in axolotls showing epidermis (E), dermis (D), hypodermis (H) and underlying muscle (M). B) Magnified image of epidermis and dermis. The epidermis is pseudo-stratified and contains epithelial cells and leydig cells (yellow arrows) while the dermis is divided into the stratum spongiosum (containing glands and dermal fibroblasts) atop the densely compacted ECM of the stratum compactum. C-H) Scar-free healing over 80 day period following full thickness excisional wounding. C) One day post injury (dpi) the wound bed is completely re-epithelialized. Some blood plasma has accumulated beneath the neoepidermis (green arrows) (wound margin = WM). D) Seven dpi there is little evidence of a fibrin clot between the epidermis and underlying muscle and no new ECM has been deposited. Green arrows depict residual blood plasma. E) Fourteen dpi fibroblasts are visible beneath the epidermis where new ECM is deposited (blue staining) and muscle cells are fragmenting into individual myoblasts. F) Twenty-one dpi a thick band of transitional matrix is visible beneath the epidermis. Collagen is visible within the regenerating muscle. G) Forty-seven dpi the underlying muscle has completely regenerated and is devoid of collagen. Skin glands (yellow arrow) have regenerated and descended from the epidermis. The dermal stratum spongiosum has reformed and the stratum compactum is coalescing. H) Eighty dpi all skin layers have regenerated. Scale bars = 100 mm.

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Curriculum Vitae

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ACADEMIC APPOINTMENTS

2013-Present	Assistant Professor, University of Kentucky, Dept. of Biology
2010-Present	Visiting Professor, University of Nairobi, Department of Veterinary Anatomy and Physiology

EDUCATION

2009-2013	Post-doctoral Fellow, **University of Florida**, Department of Biology Mentor: Malcolm Maden, Ph.D.
2004-2009	**University of Florida,** Department of Zoology Ph.D., Developmental Biology, Advisor: Martin J. Cohn, Ph.D.
2008	SDB Short Course: *Frontiers in Developmental Biology: Concepts, Techniques and Model Organisms.*
2001-2004	**University of Florida,** Department of Zoology M.Sc., Physiological Ecology, Advisor: Lauren J. Chapman, Ph.D.
1994-1998	**Bowdoin College** A.B., Biology; minor in Philosophy, concentration in Photography

PUBLICATIONS

Peer-reviewed Articles and Reviews (*denotes equal contribution)

Simkin, J. and **Seifert, A.W.** (2017). Translating regenerative biology into clinically relevant therapies: are we on the right path? *Stem Cell Translational Medicine.* http://dx.doi.org/ 10.1002/sctm.17-0213

Seifert, A.W., and Muneoka, K. (2017). The blastema and epimorphic regeneration in mammals. *Developmental Biology* 433 (2018) 190–199

Simkin, J., Gawriluk, T.R., Gensel, J., and **Seifert, A.W.** (2017). Epimorphic regeneration in spiny mice requires cellular inflammation and immunity. *eLife* DOI: 10.7554/eLife.24623.

Cook, A.B., and **Seifert, A.W.** (2016). Beryllium nitrate inhibits fibroblast migration to disrupt epimorphic regeneration. *Development* 143, 19: 3491-3505. doi: 10.1242/dev.134882

Leung, M.C.K, Hutson, M. Shane, **Seifert, A.W.**, Baker, N.C., Spencer, R.M., and Knudsen, T.B. (2016). Computational Modeling and Simulation of Genital Tubercle Development. *Reproductive Toxicology.* DOI: 10.1016/j.reprotox.2016.05.005

Armfield, B.A., **Seifert, A.W.**, Zheng, Z., Merton. E.M., Rock, J.R., Lopez C., Baker H.V., and Cohn, M.J. (2016). Molecular Characterization of the Genital Organizer: Gene Expression Profile of the Mouse Urethral Plate Epithelium. *Journal of Urology.* DOI: 10.1016/j.juro.2016.04.091

Gawriluk, T. R., Simkin, J., Thompson, K.L., Biswas, S., Clare-Salzler, Z., Kimani, J.M., Kiama, S.G., Smith, J.J., Ezenwa V.O., and **Seifert, A.W**. (2016). Comparative analysis of ear hole closure identifies epimorphic regeneration as a discrete trait in mammals. *Nature Communications.* DOI: 10.1038/ncomms11164

Haughton, C.L., Gawriluk, T.R., and **Seifert, A.W**. (2016). The African spiny mouse (*Acomys cahirinus):* biology and husbandry of a laboratory colony**.** *JAALAS.*

Gredler, M. L., **Seifert, A. W.,** and Cohn, M. J. (2015). Tissue-specific roles of Fgfr2 in development of the external genitalia. *Development* 142 (12), 2203-2212.

Maden, M., Avila, D., Roy, M., and **Seifert, A.W**. (2015). Tissue specific reactions to positional discontinuities in the regenerating axolotl limb. *Regeneration.* DOI: 10.1002/reg2.35

Gredler, M. L., **Seifert, A. W.,** and Cohn, M. J. (2014). Phallus and cloaca development in the American alligator, *Alligator mississippiensis*. *Journal of Sexual Development*, 9:53-67

Seifert, A.W., and Maden, M. (2014)**.** New insights into skin regeneration. *International Review of Cell and Molecular Biology.* 310, 129-169.

Monaghan, J. R.*, Stier, A.C.*, Michonneau, F.*, Smith, M.D.*, Pasch, B.*, Maden, M **and Seifert, A. W.** *. (2014). Experimentally induced metamorphosis in axolotls reduces regenerative rate and fidelity. *Regeneration.* 1: 1, 2-14

Seifert, A.W., and Voss, S.R. (2013)**.** *Commentary*: Revisiting the relationship between regenerative ability and aging. *BMC Biology*, **11**:2

Seifert, A.W., Kiama, S.G., Seifert, M.G., Goheen J., Palmer, T. M., and Maden, M. (2012). Skin shedding and tissue regeneration in African spiny mice (*Acomys*). *Nature,* Sep 27; 489(7417):561-5.

Monaghan, J. R., Athippozhy, A., **Seifert, A.W**., Putta, S., Stromberg, A., Maden, M., Gardiner, D. M., and Voss, S. R. (2012). Denervation quantitatively and systemically alters transcription within the wound epithelium of a regeneration-competent salamander limb. *Biology Open*, Oct 15;1(10):937-48.

Seifert, A.W., Monaghan, J.R., Voss, S.R., and Maden, M. (2012). Skin regeneration in adult axolotls: A blueprint for scar-free healing in vertebrates. *PLoSOne* 7(4):e32875. Epub, Apr 2

Seifert, A. W., Monaghan, J.R., Smith, M.D., Pasch, B., Stier, A.C., Michonneau, F., and Maden, M. (2012). The influence of fundamental traits on mechanisms controlling appendage regeneration *Biological Reviews of the Cambridge Philosophical Society*. May; 87(2):330-45, Epub 2011, Sep 19.

Seifert, A.W*., Zheng, Z*, Ormerod, B.K., and Cohn, M.J. (2010). *Shh* controls growth of external genitalia by regulating cell cycle kinetics. *Nature Communications* 1(3):1-9.

Seifert, A.W., Bouldin, C., Choi, K.S., Harfe, B.H., and Cohn, M.J., (2009). Multiphasic and tissue specific roles for *Shh* during anogenital development. *Development* 136 (23):3949-57

Seifert, A.W., Yamaguchi, T., and Cohn, M.J. (2009). Functional and phylogenetic analysis shows that *Fgf8* is a marker of genital induction in mammals but is not required for external genital development. *Development* 136(15):2643-51.

Seifert, A.W., Harfe, B.H., and Cohn, M.J. (2008). Cell lineage analysis demonstrates an endodermal origin of the distal urethra and perineum. *Developmental Biology* 1;318(1):143-52. (cover article)

Seifert, A.W., and Chapman, L.J. (2007). The relationship between temperature and standard rate of metabolism in the African lungfish *Protopterus aethiopicus*, from Uganda. *African Journal of Aquatic Sciences*, 32(3) 299-303.

Garner, S., Birt, T.P., Mlewa, C.M., Green, J.M., **Seifert, A.**, and Friesen, V.L. (2006). Genetic variation in the marbled lungfish *Protopterus aethiopicus* in Lake Victoria and introduction to Lake Baringo, Kenya. *Journal of Fish Biology*, 69: 189-199.

Seifert, A.W., and Chapman, L.J. (2006). Respiratory allocation and standard rate of metabolism in the African lungfish, *Protopterus aethiopicus*. *Comparative Biochemistry and Physiology: Molecular and Integrative Physiology*, 143(1):142-8.

Chapman, L.J., Nordlie, F.G., and **Seifert, A**. (2002). Respiratory oxygen consumption among groups of the African cichlid *Pseudocrenilabrus multicolor victoriae* subjected to different oxygen concentrations during development. *Journal of Fish Biology*, 61: 242-251.

Articles – in review

Kimani, J.M., Kiama, S.G., Towett, P.K., and **Seifert, A.W**. Morphological adaptations to subterranean life: comparative anatomy of the skin from the African mole rat (*Tachyoryctes ibeanus)* (in revision *Journal of Morphology*)

Cyr, J. L., Gawriluk, T. R., Rada, B., Wendy T. Watford, W. T., **Seifert, A. W**., and Ezenwa, V. O. (*in revision*). Comparison of neutrophils from a regeneration-competent and incompetent mammal reveals subtle differences in cell quantity and function. *Frontiers in Immunology: Comparative Immunology*.

Cook, A. B., Shaw, D. and **Seifert, A. W**. (*in revision*). Normal skin development in salamanders reveals two routes to skin regeneration. *Development*

Book Chapters, Edited Volumes, Technical Reports, Other

Seifert, A.W. (2017). Comparative Regeneration. *Developmental Cell 43*. p. 373.

Coots, P., and **Seifert, A.W**. (2015). Thyroxine induced metamorphosis in the axolotl (*Ambystoma mexicanum*). In, *Salamanders in Regeneration Research* in Methods in Molecular Biology, eds. Kumar, A. and Simon, A. Springer. USA. pp. 141-145.

Ankerson, T.T., Fernandez, S., M., **Seifert, A.W**., and Velez, M.V. (2003). The gopher tortoise and upland habitat protection in Florida: Legal and policy considerations. In, Report on protected species and habitat protection, Ankerson, T.T., Fernandez, S.M., and Seifert, A.W. (eds). Report available through UF Levin College of Law library.

GRANTS, FELLOWSHIPS AND INDUSTRY FUNDING

2017-2022	R01 NIH - NIAMS, *Macrophage phenotype orchestrates tissue regeneration in mammals* (**PI**) –
2016-2019	Fundacao de Ciencia y Tecnologia (Portugal) (**International Collaborator**) - *Regeneration in African Spiny Mice*
2015-2018	NextGen Biologics Inc. (**PI**) - *Developing an amphibian sourced surgical mesh*
2015	Igniting Research Collaboration, University of Kentucky (**Co-PI**) - Sphingolipids and delayed wound healing in diabetes.
2015	National Geographic Grants in Aid of Research (NGS) (**PI**) - *Innate immunity and regeneration in East African rodents*
2014-2018	NSF, Division of Integrated Organismal Systems (IOS) and Office of International Science and Engineering (ISE). (**PI**) – *Immune tradeoffs during tissue regeneration in mammals*
2012-2013	SEED Biotechnologies Corporation. (**PI**) – funding for technician and research into scar-free healing in axolotls
2012 (spring)	SEED Biotechnologies Corporation. (**PI**) – for continued study exploring skin regeneration in salamanders towards developing regenerative therapies in humans
2009-2013	NIH NIDDK Postdoctoral Fellowship in Regenerative Medicine NIH T32DK074367
2009-2013	Singer Biology Fund Award. (**Co-PI**) – Effects of body size, age and metamorphosis on regenerative ability in *Ambystoma mexicanum* (axolotl).
2008	Department of Zoology Research Grant.– Genetic control of external genital development in mammals.
2008	HHMI GATOR Research Award. – Fibroblast growth factor expression during external genital development.
2001-2004	Grinter Fellowship. – Graduate fellowship awarded by University of Florida to cover summer research expenses
2003	National Science Foundation GRF (Honorable mention)
2002	Raney Fund, ASIH. – Metabolism across ontogeny in the African lungfish, *Protopterus aethiopicus.*
2002	Sigma Xi Grants in Aid of Research.) -- Energetics of the African lungfish, *Protopterus aethiopicus*

AWARDS

2011	Southeast Society for Developmental Biology Meeting -- Best Talk – awarded travel to SDB main meeting
2008	Society for Developmental Biology -- Best Poster, SDB Short Course (Runner-Up)

TEACHING

2017	MDBIL Summer Course: Comparative Regenerative Biology (Instructor)
2017 - ongoing	BIO 429/629: Developmental Biology (every spring)
2016	BIO 199: Stemcats Research Experience
2015	BIO 425: Animal Development and Regeneration
2015 - ongoing	UKC 300/A&S 300 BioArt: Creativity, Experimentation and Methodology in Art and Science (every other spring)
2014	BIO 197 StemCats (Faculty Participant)
2014 - ongoing	BIO 770 Topics in regeneration biology (every Fall)
2014	BIO 770 Principles of Regeneration
2014	BIO 580 Stem cells, tissue engineering and regenerative medicine (Co-Instructor)

2012	Seminar in Regeneration Biology (instructor)
2011	Integrative Principles (guest lecturer)
2011-2013	Biology for Non-majors (guest lecturer)
2010-2011	Evolution of Regenerative Mechanisms (co-instructor)
2008-2010	Reproductive Biology (guest lecturer)
2009	Seminar in The Cell Cycle and Development (instructor)
2007-2008	Howard Hughes Mentor
2004-2005	Developmental Biology (teaching assistant)
2004-2005	Genes, Embryos and Evolution (teaching assistant)
2001-2004	Functional Vertebrate Anatomy (laboratory instructor)

MENTORING

Postdoctoral Scholars

Tom Gawriluk (2014-present). Department of Biology, University of Kentucky (Mentor)
Jennifer Simkin (2015-present). Department of Biology, University of Kentucky (Mentor)

Current

Graduate Students (Advisor)

Sandeep Saxena (2016-present). Ph.D student. Department of Biology, University of Kentucky
Sruthi Purushothaman (2014-present). Ph.D student. Department of Biology, University of Kentucky
Shishir Biswas (2014-present). Ph.D student. Department of Biology, University of Kentucky
John Kimani (2014-present). Ph.D student. Department of Veterinary Anatomy and Physiology, University of Nairobi (**Co-Advisor**)
Luc Dunoyer (2013-present). Ph.D. student. Department of Biology, University of Kentucky (**Co-Advisor**)

Graduate Students (Committee Member)

Kristyn Van Der Meulen (2017-present). Ph.D. student. Department of Biology, University of Kentucky
Alex Chen (2015-present). Ph.D. student. Department of Biomedical Engineering, University of Kentucky
Nicholas Kohrs (2015-present). M.Sc student. Department of Biomedical Engineering, University of Kentucky
Chanung Wang (2014-present). Ph.D. student. Department of Biology, University of Kentucky

Completed

John Kimani (2010-2013). M.Sc. Department of Veterinary Anatomy and Physiology, University of Nairobi. *Wound healing and regeneration in the naked mole rat, Heterocephalus glaber.* (**Co-Advisor**).
Tom Gawriluk (2013-2014). Ph.D. Candidate. Department of Biology, University of Kentucky. *Targeted knockout of Beclin-1 reveals an essential function in ovary and testis.* (**Committee Member**).
Adam Cook (2013-2015). M.Sc Candidate. Department of Biology, University of Kentucky. *Beryllium nitrate supports fibroblast migration as an essential component of skin and limb regeneration in axolotls.* (**Advisor**)
Peggy Coots (2013-2015). MFA Candidate, Department of Fine Arts, University of Kentucky. (**Committee Member**).
Stephanie Bryant (2014-2016). Ph.D. Candidate. Department of Biology, University of Kentucky (**Committee Member**).

Undergraduate Students

Current

Corin Hacker (2015-present). Cartilage regeneration in African spiny mice.

Completed (*indicates publication with student)

Madison Ginn (2017). Genetic basis of salamander limb development.
Aaron Hoback (2016-2017). Immunity and regeneration in African spiny mice.
Cassandra Wooley (2016-2017). Requirement for macrophages during regeneration
Malik Guidry (2014-2016). Molecular basis of pattern formation. NSF REU student
Bailey Gensheimer (2015-2016). Effect if stress induced glucocorticoids on tissue regeneration.
Juliana Lavey (2014-2016). Molecular basis of pattern formation.
*Douglas Shaw (2013-2016). Skin microbiome axolotls. (BIO 395 student)
Zara Ashraf (2013-2015). Blastema formation during spiny mouse ear hole regeneration. (BIO 395 student)
Gavin Hall (2013-2014). Wnt/ß-catenin signaling during skin development and regeneration in axolotls.
Oliver Olivarez (2012-2013). Role of ECM during skin regeneration in salamanders.
Kirabet Quintana-Ramos (2012, summer). MMP function during skin regeneration in metamorphic axolotls.
*Daima Avila (2012-2013). Ear hole regeneration in mammals.
Adam Cook (2012-2013). Evolution of regenerative mechanisms in Urodeles. (Graduate student in Biology, UK).
Natalie Nelson (2011-2012). Skin development in *Ambystoma mexicanum*.
Joshua Hochberger (2010-2012). Identifying developmental mechanisms regulating wound healing and regeneration in Anurans.
Conner Burnside (2010-present). Developmental biology of the integument in *Ambystoma mexicanum*
Sean Connelly (2010-2012). The effects of functional traits on regenerative ability in *Ambystoma mexicanum*
McKayla Spencer (2010-2011). The effects of functional traits on regenerative ability in *Ambystoma mexicanum*
Jenny Fernandez (2008-2009). OPT imaging of the murine external genitalia.
Liz Dantzler (2008-2009). Fgf signaling during sexual differentiation of the external genitalia. (*publication in prep, see above*).
Rickesh Patel. (2007-2009). Howard Hughes Mentoring Program. Development and cellular origin of the mammalian vagina.
*Marissa Gredler (2007-2008). Functional analysis of *FgfR2* in the genital tubercle.
Stacey Gray. (2007). Howard Hughes Mentoring Program. *Foxa1* and *Foxa2* regulation of perineum formation.
Christina Varvarikos. (2006-2007). Howard Hughes Mentoring Program. Fgf signaling during genital development.
Virginia Hoglund (2005). Histological analysis of the developing mouse genital tubercle.
Eric Rubin (2005-2007). Androgen signaling during mammalian urethragenesis.
Sarah Tanaka (2003). Analysis of Q_{10} measures in an air-breathing fish, *Protopterus aeithiopicus*
Michael Wasserman (2002). Aestivation in the lungfish, *Protopterus aeithiopicus*.

ORAL PRESENTATIONS

2018 Invited Speaker: *TBD,* The Molecular & Cellular Basis of Regeneration and Tissue Repair, Malta
2018 Invited Speaker: *TBD,* Frontiers in Veterinary Medicine Speaker Series, University of Calgary, Calgary

2018 Invited Speaker: Spiny mice and complex tissue regeneration in mammals, Smith College, Northampton, MA

2017 Invited Speaker: *African spiny mice as an emerging model of epimorphic regeneration in mammals*, CDB Symposium: Mavericks in Developmental Biology, RIKEN Center for Developmental Biology, Kobe, Japan

2017 Invited Speaker: *African spiny mice, tissue regeneration and the injury microenvironment*, Cornell University, Department of Molecular Medicine, Seminar Series, Ithaca, NY

2017 Invited Speaker: *Unraveling the mechanistic basis for regeneration in spiny mice*, SDB 75th Annual Meeting, Organogenesis and Regeneration Session, Minneapolis, MN

2017 Invited Speaker: *Using spiny mice to reveal cellular regulators of tissue regeneration*, University of Southern California, Department of Stem Cell Biology and Regenerative Medicine, Distinguished Speaker Seminar Series, Los Angeles, CA.

2017 Invited Speaker: *TBD*, Vanderbilt University Medical Center (VUMC), Pathology, Microbiology and Immunology (PMI) Seminar Series, Nashville, TN (cancelled)

2016 Invited Speaker: *Immune cell dynamics during epimorphic regeneration in spiny mice*, Medical University of South Carolina (MUSC), Department of Regenerative Medicine and Cell Biology, Charleston SC.

2016 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Stem Cell Society Singapore (SCSS), Symposium 2016: Modeling Cell Fate and Development, Biopolis, Singapore (did not attend)

2016 Invited Speaker: *Immune cell dynamics during epimorphic regeneration in spiny mice.* Max Plank Institute for Molecular Biomedicine, Muenster Germany.

2016 Invited Speaker. *Blastema formation and musculoskeletal regeneration in mammals.* Weldon School of Biomedical Engineering, Sept. 28th 2016.

2016 Invited Speaker: *Animal regeneration and development*, FASEB Educational Briefing, Capital Hill, Washington, D.C.

2016 Invited Speaker: *Epimorphic regeneration in African spiny mice.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA

2016 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Pasteur Institute, Department of Developmental and Stem Cell Biology, Paris, France

2015 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Wakeman Institute, Rutgers University, Departmental Seminar Series, Piscataway, NJ

2015 Invited Speaker: *Integrating art and biology: in the classroom, the laboratory and beyond.* George Church Laboratory, Department of Genetics, Harvard University, Cambridge, MA

2015 Invited Speaker: *Epimorphic regeneration in African spiny mice*, Harvard Department of Stem Cell and Regenerative Medicine, Cambridge, MA

2015 Invited Speaker: *Comparative biology of vertebrate regeneration*, MDI Biological Laboratory, Symposium: Comparative Biology of Tissue Repair, Regeneration and Aging, Salisbury Cove, ME

2015 Invited Speaker: *Regeneration: From Axolotls to Acomys*, Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

2015 Invited Speaker: *Comparative biology of vertebrate regeneration*, Department of Biomedical Engineering, University of Kentucky, Lexington, KY

2015 Invited Speaker: *Complex tissue regeneration in African spiny mice*, Department of Craniofacial and Mesenchymal Biology, UCSF, San Francisco, CA

2014 Invited Speaker: *New insights into mammalian skin regeneration*
Department of Cell and Molecular Biology, Tulane University, New Orleans, LA

2014 Invited Speaker: *Understanding limitations on vertebrate organ regeneration.*
CDB Symposium, RIKEN: Regeneration of Organs: Programming and Self-Organization, Kobe,

Japan

2012 Invited Speaker: *Weak skin, spiny hairs and regeneration in African spiny mice (Acomys).* Department of Biology, Texas Tech University.

2012 Invited Speaker: *Skin shedding and tissue regeneration in African spiny mice (Acomys).* Keystone Meeting; Mechanisms of Whole Organ Regeneration, Breckenridge, CO.

2011 Invited Speaker: *Perfect Skin Regeneration: New Models for Scar-Free Healing.* Department of Biomedical Sciences, Cornell University.

2011 Selected Speaker: *Skin regeneration in adult axolotls: A blueprint for scar-free healing in vertebrates.* Southeast Society for Developmental Biology, University of Florida.

2010 Invited Speaker: *Development of the anogenital system in mammals.* University of Nairobi, Department of Veterinary Anatomy and Physiology.

2007 Selected Speaker: *Origin of the penile urethra and the proximodistal fate of the urethral epithelium.* Society for Developmental Biology, Cancun Mexico.

2004 Selected Speaker: *Respiratory allocation and the standard rate of metabolism in the African lungfish, Protopterus aethiopicus.* Society for Experimental Biology main meeting, Edinburgh, Scotland.

2003 Invited Speaker: *The gopher tortoise and upland habitat protection in Florida: Legal and policy considerations.* Protected Species and Upland Habitat Workshop, UF Levin College of Law, Gainesville, Florida.

UNIV. OF KENTUCKY PRESENTATIONS

2017 Regenerative Biology at UK, A&S College Donor's Dinner, University of Kentucky

2017 SCoBIRC Works in Progress Seminar Series, University of Kentucky

2014 Research in Animal Regeneration: STEMCATS Chat, University of Kentucky

2013 Models in Regeneration Biology, Dean's Circle, University of Kentucky

POSTER PRESENTATIONS

2017 Purushothaman, S. and **Seifert, A.W.** *Understanding urodele limb development in the context of tetrapod limb evolution.* 14th International Conference on Limb Development and Regeneration, July 23rd, 2017, Edinburgh, Scotland

2016 Biswas, S., and **Seifert, A.W**. *Mammalian blastema formation in the African spiny mouse.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2016 Purushothaman, S. and **Seifert, A.W.** *Understanding urodele limb regeneration in the context of tetrapod limb development.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2016 Gawriluk, T. R., Simkin, J., Thompson, K.L., Biswas, S., Kiama, S.G., Ezenwa V.O., and **Seifert, A.W**. Understanding the immune response to injury in a regenerating mammal (*Acomys spp.*). SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2016 Simkin, J.S., Gensel J.C., and **Seifert A.W.** *Inflammation: setting the stage for tissue regeneration.* SDB 75th Annual Meeting Satellite Symposium: Evolution of regenerative abilities, August 4th, 2016, Boston MA.

2015 Simkin, J.S., Gawriluk T., Biswas S., Gensel J.C., and **Seifert A.W.** *Macrophage phenotype reveals fundamental differences between regeneration and scarring.* MDI Biological Laboratory, Symposium: Comparative Biology of Tissue Repair, Regeneration and Aging, Salisbury Cove, ME

2015 Cook, A.B., and **Seifert, A.W.** *Beryllium nitrate reveals fibroblast migration as an essential*

component of skin and limb regeneration in axolotls. MDI Biological Laboratory, Symposium: Comparative Biology of Tissue Repair, Regeneration and Aging, Salisbury Cove, ME

2010 **Seifert, A.W.,** Monaghan, J., and Maden, M. Skin regeneration in the Mexican axolotl *(Ambystoma mexicanum)*. Florida Genetics, Gainesville, Florida.

2010 **Seifert, A.W.,** Monaghan, J., and Maden, M. Skin regeneration in the Mexican axolotl *(Ambystoma mexicanum)*. EMBO Meeting; Molecular and cellular basis of tissue repair and regeneration, Sesimbra, Portugal.

2010 Monaghan, J., **Seifert, A.W.**, and Maden, M. Nerve-dependent gene expression in the epidermis of regenerating salamander limbs. EMBO Meeting; Molecular and cellular basis of tissue repair and regeneration, Sesimbra, Portugal.

2009 **Seifert, A.W.**, Ormerod, B.K., and Cohn, M.J. *Sonic hedgehog* regulates cell cycle kinetics during morphogenesis. Society for Developmental Biology 68th Annual Meeting, San Francisco, California.

2009 **Seifert, A.W.**, Yamaguchi, T., and Cohn, M.J. Functional and phylogenetic role of *Fgf8* during external genital development. Society for Developmental Biology 68th Annual Meeting, San Francisco, California.

2008 **Seifert, A.W.**, Ormerod, B.K., and Cohn, M.J. *Sonic hedgehog* regulates cell cycle kinetics during morphogenesis. Latin American Society for Developmental Biology, Buenos Aires, Argentina.

2007 **Seifert, A.W.**, Harfe, B, and Cohn, M.J. Multiphasic role of *Shh* during external genital development and cloacal septation. Gordon Research Conference; Developmental Biology, New London, New Hampshire.

2007 **Seifert, A.W.**, Harfe, B, and Cohn, M.J. Multiphasic role of *Shh* during external genital development and cloacal septation. Society for Developmental Biology 66th Annual Meeting, Cancun, Mexico.

2006 **Seifert, A.W.**, Harfe, B, and Cohn, M.J. Origin of the penile urethra and the proximodistal fate of the urethral epithelium. Society for Developmental Biology 65th Annual Meeting, Ann Arbor, Michigan.

CONSULTING POSITIONS

2015-present	Consultant, NeXtGen Biologics, Inc.
2011-present	Consultant, ISE Professional Testing and Consulting Services, Inc.
2010-2014	Scientific Advisory Board, Regenerative Medicine International

PROFESSIONAL AFFILIATIONS

Society for Developmental Biology

SERVICE

2017-ongoing	Univ. of Kentucky, International Partnerships and Research Committee
2017	Univ. of Kentucky Faculty Search Committee (Stem Cells and Reg. Biol.)
2016-ongoing	Univ. of Kentucky, Dept. of Biology Executive Committee
2016-ongoing	Society for Developmental Biology (SDB) Public Affairs Committee
2016	FASEB Educational Briefing to Congressional Members and Staff
2016	Co-Organizer, SDB 75th Annual Meeting Satellite Symposium on Regeneration
2014-2015	National Science Foundation (NSF) Reviewer
2015-ongoing	Univ. of Kentucky, Dept. of Biology, Seminar Committee
2013-2015	Univ. of Kentucky Graduate Affairs Committee (GAC)
2015	Univ. of Kentucky Faculty Search Committee (Int. Physiologist Faculty Hire)

2013	Univ. of Kentucky Faculty Search Committee (Dev. Biol. Faculty Hire)
2011-2013	Univ. of Florida Postdoctoral Affairs Advisory Committee
2012-2013	Postdoctoral Mentoring Program
2005	Dept. of Biology, Faculty Search Committee (Evolutionary Functional Morphology)
2004	Graduate Curriculum Committee
2007-2008	Vice President, Zoology Graduate Student Association

Granting Agencies – Ad Hoc Reviewer
National Geographic Society, Department of Defense (ARO), National Science Foundation (NSF), Swiss National Science Foundation (SNF)

Academic Journals – Ad Hoc Reviewer
Science Reports, Developmental Cell, PLosOne, Journal of Morphology, EvoDevo, Journal of Sexual Medicine, Faculty of 1000, JoVE, Regeneration, JEZ Part B: Molecular and Developmental Evolution, Development, Journal of Anatomy, Developmental Dynamics, BMC Biology, BMC Developmental Biology, Neurogenesis, Biology of Reproduction

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	1	of	1	GROUP ART UNIT: 1651	EXAMINER: R. A. Davis

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		WO	2008/129997	A1	School Juridical Person Kitasato Gakuen	10-30-2010	Y

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		SATOH et al., Collagen reconstitution is inversely correlated with induction of limb regeneration in Ambystoma mexicanum. Zoolog Sci. 2012 Mar;29(3):191-7. doi: 10.2108/zsj.29.191.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/RUTH A DAVIS/	04/12/2018

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.A.D/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01	3358

23628 7590 04/20/2018

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BOSTON, MASSACHUSETTS 02210-2206
UNITED STATES OF AMERICA

EXAMINER
DAVIS, RUTH A

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
04/20/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 14/759,895	Applicant(s) Early, Ryanne	
	Examiner RUTH A DAVIS	Art Unit 1651	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 3/14/2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-33,36-38 and 40-61 is/are pending in the application.

5a) Of the above claim(s) 1-4,6-19,23-25 and 27-33 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 5,20-22,26,36-38 and 40-61 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Applicant's amendment, reply and affidavit filed July 25, 2017 have been received and entered into the case. Claims 34, 35 and 39 are canceled; claims 49 – 61 are added. All arguments and the affidavit have been fully considered.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 42 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

In claim 42, the phrase "an agent found in a human" is not adequately defined by the claim language or specification, thus the scope of the claim has not been clearly delineated.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.

Claims 5, 20 - 22, 36 - 38, 41, 44 and 47are rejected under 35 U.S.C. 102a1 as being anticipated by Satoh et al. (2012).

Satoh teaches acellular collagen and ECM sheets (decellularized ECM) from axolotl (p.192). Although Satoh does not teach each of the claimed process steps by which the collagen is produced, these limitations are considered to product by process type limitations. It is noted that the patentability of a product does not depend on its method of production. If the claimed product is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference), the claim is unpatentable even though the reference product was made by a different process. When the prior art discloses a product which reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C 102 and/or 35 U.S.C 103 are proper. (MPEP 2113) Regarding the intended use of decellularizing the ECM to retain structure and functional integrity of the ECM, this intended use of the claimed composition does not patentably distinguish the composition, per se, since such undisclosed use is inherent in the reference composition. In order to be limiting, the intended use must create a structural difference between the claimed composition and the composition of the prior art. In the instant case, the intended use fails to create a structural difference, thus, the intended use is not limiting. Please note that when applicant claims a composition in terms of

function, and the composition of the prior art appears to be the same, the Examiner may make rejections under both 35 U.S.C 102 and 103 (MPEP 2112). Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen.

Therefore, the reference anticipates the claimed subject matter.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 5, 20 – 22, 26, 36 – 38, 40 - 61 are rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

Asem teaches decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col.5 line 23-39), derived from amphibians (col.4 line 35-40). The ECM may in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col.6 line 43-64). The ECM compositions may be used as tissue grafts constructs (col.4 line 30-35) or with cell culture comprising growth factors for culturing human (col.11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col.7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45).

Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl. However, at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration.

Thus, the claims are rendered obvious.

Response to Arguments

Applicant argues that Satoh does not teach the claimed ECM retaining its structure and function or having reduced antigenicity; that it is not the same or similar to the claimed ECM in that the ECM of Satoh is crosslinked with glutaraldehyde whereas the claimed ECM requires different process steps. Applicant argues the claimed graft requires removing cellular debris and no crosslinking whereas Satoh uses miniscule amounts of ECM. Applicant argues the examiner cannot prove the ECM of the prior art eliminated antigenicity or is suitable for xenograft and that the intended use and product by process limitations results in structural differences which are patentable. Applicant further argues that there is no sound reasoning to the combination of Asem

and Seifert; that examiner uses improper hindsight; that Seifert teaches away from using ECM for healing; that Asem does not identify functional equivalents or the use of ECM in a xenograft. Applicant provides a declaration stating the same.

However, these arguments fail to persuade.

Regarding Satoh the reference teaches a decellularized ECM sheet from a Urodele as claimed. The reference teaches an acellular ECM, or decellularized, which would inherently have the claimed retention of structural and functional integrity as claimed. Even if the ECM is crosslinked with glutaraldehyde, it would still retain sufficient structure and function to permit and support use of the matrix as a substrate for growth of cells in vivo or in vitro, as defined by the instant specification on page 12, thus inherently meeting the claimed limitations. Absent clear evidence of a structural or functional difference between the claimed ECM and that of Satoh, the arguments are found unpersuasive.

Regarding the argument that the prior art does not teach the claimed process steps, it is noted that the claim fails to recite active process steps as argued. Rather, the claims only recite functional descriptive language, without any specific step to meet the limitations. The claims do not require any step for removing cellular debris, any amount of ECM, nor do they exclude crosslinking. Thus, the argument is not commensurate in scope with the claimed invention.

Regarding applicant's assertion that the examiner has not proven the ECM of the prior art has eliminated antigenicity and is suitable for xenograft, it is maintained that the prior art teaches acellular collagen, acellular ECM, and decellularized basement membranes, which means the ECM are free of cellular components. As defined by applicant on page 12, being free of cells and cellular components eliminates or reduces antigenicity as well as makes the material suitable for xenograft. Further, as the claimed invention fails to recite any specific step for making the

graft, the claims neither require nor exclude any step in achieving such a decellularized ECM. As such, the claims read on the prior art. It is noted that the burden is on applicant to provide arguments and evidence establishing that the claimed product is different from that of the prior art, and that such arguments and evidence must be commensurate in scope with the claimed invention.

Regarding the intended use of the compositions, it is noted that intended use of the claimed composition does not patentably distinguish the composition, per se, since such undisclosed use is inherent in the reference composition. In order to be limiting, the intended use must create a structural difference between the claimed composition and the composition of the prior art. In the instant case, the intended use fails to create a structural difference, thus, the intended use is not limiting. Please note that when applicant claims a composition in terms of function, and the composition of the prior art appears to be the same, the Examiner may make rejections under both 35 U.S.C 102 and 103 (MPEP 2112). Furthermore, the claimed process does not recite any active steps that must be practiced to arrive at a product with structural or physical differences. It is reiterated that the claims only recite functional descriptive language, without any specific step to meet the limitations. As such, the claims encompass any decellularized ECM with structural/functional integrity and has been decellularized. Thus, the argument is not commensurate in scope with the claimed invention.

Regarding the combination of Asem and Seifert, it is noted that it is the combination of references that is relied upon. Asem teaches decellularized ECM derived from amphibians in the form of sheets that can be adhered together (or laminated), powderes (or particles) or reconstituted gels, which are made under alkaline conditions. Asem teaches the ECM compositions may be used as tissue grafts constructs, with cell culture, or culturing human cells

wherein it is preferably sterilized prior to use. Seifert is relied upon to demonstrate that Urodele, specifically axolotl, was a known amphibian source from which a decellularized ECM may be obtained. Moreover, Seifert provides evidence of a functionally equivalent amphibian source of ECM that can be decellularized and used in the compositions of Asem with a reasonable expectation for success.

Regarding a teaching away, it is acknowledged that Seifert teaches different outcomes than applicant. However a different outcome is not a teaching away from functionality of the ECM. Seifert is not cited to motivate one in the art to use a crosslinked ECM from Urodele in the compositions of Asem, but rather to evidence it was a known source of amphibian ECM that can be decellularized and used as the amphibian ECM in the compositions of Asem with a reasonable expectation for success. Please note that the instant claims do not exclude or require any step for producing the decellularized ECM, nor do they require or exclude any particular type or source of ECM other than being any tissue from a Urodele. Thus, the arguments are not commensurate in scope with the claimed invention.

In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. See In re McLaughlin, 443 F.2d 1392, 170 USPQ 209 (CCPA 1971).

Conclusion

All claims are drawn to the same invention claimed in the application prior to the entry of the submission under 37 CFR 1.114 and could have been finally rejected on the grounds and art of record in the next Office action if they had been entered in the application prior to entry under 37 CFR 1.114. Accordingly, **THIS ACTION IS MADE FINAL** even though it is a first action after the filing of a request for continued examination and the submission under 37 CFR 1.114. See MPEP § 706.07(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH A DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on Monday - Friday (8am - 4pm).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUTH A DAVIS/
Primary Examiner, Art Unit 1651

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14/759,895	Early, Ryanne
	Examiner	Art Unit
	RUTH A DAVIS	1651

CPC - Searched*		
Symbol	Date	Examiner
A61K 35/65	04/15/2018	rad

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
east, pubmed, google patent	04/15/2018	rad
A61K 35/65	04/15/2018	rad

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

	/RUTH A DAVIS/ Primary Examiner, Art Unit 1651

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Request for Continued Examination (RCE) Transmittal		
Address to: Mail Stop RCE Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Application Number	14/759,895-Conf. #3358
	Filing Date	July 8, 2015
	First Named Inventor	Ryanne Early
	Art Unit	1651
	Examiner Name	R. A. Davis
	Attorney Docket Number	S1860.70000US01

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C 371, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO on page 2.)

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).
   - a. [ ] Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
     - i. [ ] Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
     - ii. [ ] Other _____
   - b. [X] Enclosed
     - i. [X] Amendment/Reply
     - ii. [ ] Affidavit(s)/ Declaration(s)
     - iii. [X] Information Disclosure Statement (IDS)
     - iv. [X] Other Expert Declaration Under 37 C.F.R. 1.132
2. Miscellaneous
   - a. [ ] Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   - b. [ ] Other _____
3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   - a. [ ] The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 23/2825 .
     - i. [X] RCE fee required under 37 CFR 1.17(e)
     - ii. [X] Extension of time fee (37 CFR 1.136 and 1.17)
     - iii. [ ] Other _____
   - b. [ ] Check in the amount of $ _____ enclosed
   - c. [X] Payment by credit card ~~(Form PTO-2038 enclosed)~~

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Edward R. Gates/	Date	October 22, 2018
Name (Print/Type)	Edward R. Gates	Registration No.	31,616

~~CERTIFICATE OF MAILING OR TRANSMISSION~~

~~I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.~~

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: October 22, 2018 Electronic Signature for: /Eileen M. MacKenzie/

6704897.1

REMARKS

This paper is being filed to accompany a Request for Continued Examination in response to the final Office Action, dated 04/20/2018 in the subject application. A Petition and Fee for a Three-Month Extension of Time is likewise provided with this Response and RCE.

Claims 1-4, 6-19, and 23-25 and 27-33 stand withdrawn from consideration as being drawn to non-elected subject matter. Rejoinder of method claims upon allowance of composition of matter claims is requested.

Claims 34, 35, and 39 are cancelled or have been previously cancelled.

Accordingly, the claims under consideration in this application prior to entrance of this Amendment were claims 5, 20-22, 26, 36-38, and 40-61; new claims 62-65 are introduced herein above.

New Claims 62-65, directed to a process of decellularization that does not involve cross-linking, are newly presented as a species of the claims from which they depend, explicitly excluding cross-linked product, and it is respectfully urged that the cited art, namely Satoh, Seifert, Asem, whether cited alone or in any reasonable combination, do not/does not teach or suggest such a process or resultant product and does not meet the positively recited limitations of these claims in which cross-linked material is excluded.

It is noted that throughout the entire specification there is significant written description and enabling disclosure regarding Urodele ECM, how to obtain it and how to decellularize it for purposes of this invention and claims (see, especially, e.g. paragraphs 0023-0040, the figures, their legends, paragraphs 0058-0092, 0094-0107 and the claims) with substantial emphasis on retention of natural architecture and function (which cross-linking constrains), without a single mention of a process including cross-linking or cross-linking agents..

It is urged that one of ordinary skill in the art would read the claims to exclude a cross-linked product and, as explained below, why the claims as amended must be read to exclude a cross-linked product.

There is but one mention of the concept of cross-linking in the entire patent disclosure - namely, at paragraph 0093 of US2015/0352257 (the USPTO's publication of the subject application). That mention does not relate to achieving the material of the present invention but

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instead relates to a use to which the material of the invention may be directed. It is quoted below in its entirety with bolded emphasis added:

"Urodele decellularized ECM can be used also for sustained delivery of therapeutic molecules, proteins or metabolites, to a site in a host. See, for example, U.S. 2004/0181240, which describes an amniotic membrane covering for a tissue surface which may prevent adhesions, exclude bacteria or inhibit bacterial activity, or to promote healing or growth of tissue, and **U.S. Pat. No. 4,361,552, which pertains to the preparation of cross-linked amnion membranes and their use in methods for treating burns and wounds. The ECMs of the invention can be used in the same manner**."

In light of the above, it is respectfully urged that whatever the fate of the of the remaining pending claims in this application, claims 62-65 should be recognized as being free of the prior art.

I. 35 USC §112:

Claim 42 stands rejected under 35 U.S.C. §112(b) or pre-AIA 35 U.S.C. §112 second paragraph, as being indefinite. Claim 42 is rejected based on the phrase "a human agent" which is asserted to not be adequately defined by the claim language or specification.

To address and overcome this ground for rejection of claim 42, the claim was previously amended to recite "an agent found in a human, whether isolated, synthetically or recombinantly produced". This text is supported by the specification at paragraph 0029 which discloses: "a human agent (i.e., an agent found in a human, isolated, synthetically or recombinantly produced)." The claim has, however, been rejected again for indefiniteness. Since claim 42 merely narrows the claim from which it depends, claim 42 is herein amended to delete the "human agent" text to overcome this ground for rejection. Reconsideration and withdrawal of this ground for rejection is therefore respectfully requested. The withdrawal of rejections for indefiniteness of Claims 44 and 61 is gratefully acknowledged.

II. 35 USC §102 and 35 USC §103:

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Claim(s) 5, 20-22, 36-38, 41, 44, and 47 stand rejected under 35 USC 102al as being anticipated by Satoh et a1. (2012), and claims 5, 20-22, 26, 36-38, and 40-61, stand rejected under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012).

It is noted for the record that Claims 26, 40, 42-43, 45-46, and 48-61 are not rejected as anticipated by Satoh, and are therefore free of this ground for rejection, and are therefore only subject to the 35 U.S.C. 103 rejection discussed below as to obviousness in light of the combination of Asem and Seifert.

With respect to novelty and non-obviousness of the claims in this application, prior to introduction of this Amendment, arguments and Declaratory evidence in support of the patentability of the claims of record and under examination have been presented, essentially as follows:

1. t is unreasonable to assert that the prior art product "appears to be **identical with** or **slightly different than** the claimed product-by-process" product;

2. the intended use, and method of producing the currently claimed product, **does** create structural differences between the claimed composition and the composition of the prior art; and

3. the Declaration of Dr. Ashley Seifert supports the assertion that significant structural and functional differences exist between the claimed ECM and the product produced by Satoh as a result of the different methods of production.

In the outstanding Final Rejection, the Examiner has essentially reiterated the anticipation rejection previously made of record, stating that:

"Satoh teaches acellular collagen and ECM sheets (decellularized ECM) from axolotl (p192). Although Satoh does not teach each of the claimed process steps by which the collagen is produced, these limitations are considered to product by process type limitations. It is noted that the patentability of a product does not depend on its method of production. If the claimed product

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is the same or obvious from a product in the prior art (i.e. the product disclosed in the cited reference), the claim is unpatentable even though the reference product was made by a different process. When the prior art discloses a product which reasonably appears to be identical with or slightly different than the claimed product-by-process, rejections under 35 U.S.C. 102 and/or 35 U.S.C. 103 are proper. (MPEP 2113) Regarding the intended use of decellularizing the ECM to retain structure and functional integrity of the ECM, this intended use of the claimed composition does not patentably distinguish the composition, *per se*, since such undisclosed use is inherent in the reference composition. In order to be limiting, the intended use must create a structural difference between the claimed composition and the composition of the prior art. **In the instant case, the intended use fails to create a structural difference, thus, the intended use is not limiting.** Please note that when applicant claims a composition in terms of function, and the composition of the prior art appears to be the same, the Examiner may make rejections under both 35 U.S.C. 102 and103 (MPEP 2112). Regarding claim 22, the claim reads on the skin and cartilage samples containing the collagen, which is considered to be a material impregnated with or having attached thereto, the collagen. Therefore, the reference anticipates the claimed subject matter."

With respect to the obviousness rejection directed to claims 5, 20-22, 26, 36-38, and 40-61 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012), the Examiner has reiterated the rejection previously made of record, notwithstanding that in the prior response, the Applicant argued and presented Declaratory evidence in support of the following arguments:

1. Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl;

2. the Seifert et al. reference, if anything, teaches away from the combination proposed by the Examiner, and this was confirmed by Dr. Ashley Seifert in the Expert Declaration filed with the prior response;

3. the Declaration of Dr. Seifert establishes that Asem teaches, if anything, that follicular tissue, like placental tissue, **is unique**, found in small quantities and is relatively difficult to obtain from eggs, including eggs of reptiles, and one would not substitute the material of Seifert for that

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of Asem. In addition, Seifert teaches that one would want to avoid the presence of ECM in promoting scar-free healing, teaching away from the combination suggested by the Examiner; and

4. Seifert's mention of ECM is all in the context of *in vivo* modelling of ECM production as it relates to scar tissue formation and there is no disclosure or suggestion in Seifert of production of anything like the ECM disclosed and claimed in the present application, and, therefore, much like the *ex vivo* tissue produced by Satoh, the Seifert material fails to meet the present claims' requirements of decellularization and reduced antigenicity.

Applicant believes that its prior showing was adequate to rebut the position taken by the Examiner, particularly when considering the Declaration by the very author of the paper cited by the Examiner, which author disagrees with the Examiner's interpretation and conclusions. Nonetheless, Applicant has further amended the claims to clearly distance the present claimed material from the material of the prior art.

III. CURRENT STATUS OF THE CLAIMS AS HEREIN AMENDED, COMPRISING (1) QUOTATION OF THE EXAMINER'S REASONS FOR MAINTAINING THE PRIOR-ART-BASED REJECTIONS AND (2) REBUTTAL REMARKS RELATING TO NEWLY INTRODUCED AMENDMENTS TO THE PENDING CLAIMS

The application provides:

"Decellularized ECM" means extra cellular matrix sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix to an extent where the matrix would be considered **non-toxic as a xenograft**."

"Non-toxic" means that a composition, **when implanted in a subject, causes little or no adverse reaction or substantial harm to cell**s and tissues in the body, and does not cause a substantial adverse reaction or substantial harm to cells and tissues in the body, for instance, the composition **does not cause** necrosis, an infection, **or a substantial immune response** resulting in harm to tissues from the implanted or applied composition."

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Applicant cites to a reference, made of record in the attached PTO1449 IDS submission, in which it is concluded that **glutaraldehyde-treated bovine pericardium incites significant toxic, inflammatory, immune, and calcification responses** which is not desirable for a long-term function as an implant. Applicant notes that Satoh uses **glutaraldehyde** in Satoh's process, which would result in a material that "incites significant toxic, inflammatory, immune, and calcification responses". Such a process and the resulting product is excluded from the claims as amended.

As amended, claim 5 provides:

A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce **decellularized extracellular matrix** by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

Decellularized extracellular matrix, as explicitly defined in the specification, **means** extra cellular matrix sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix **to an extent where the matrix would be considered non-toxic as a xenograft**.

Setoh teaches (and Applicant will shortly file a Declaration that confirms that Satoh teaches) use of **glutaraldehyde** and Satoh does not remove sufficient cellular components or reduce antigenicity to an extent where the matrix would be considered **non-toxic as a xenograft**. There are present in the material made by Satoh: DNA, glands, and nuclei. All these things are antigenic. In addition, the newly cited reference, Umashankar (and Declaration to be submitted) will confirm that **glutaraldehyde treatment of a graft material will result in a product that incites significant toxic, inflammatory, immune, and calcification responses.** Such a product is outside the scope of the claims as amended.

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In contrast, there is evidence in the application to establish the disclosed and claimed product is free of cellular debris. Figure 5 of the published patent application, US2015/0352257, shows that the claimed product has no intact glands of the dermis and all cellular constituents are removed. Applicant believes the present claims address and overcome the reasons previously stated on the record as to why the claimed invention is anticipated and/or obvious in view of the cited art.

For the sake of completeness, the Examiner, at pages 5-8 of the outstanding Final Rejection, provides the following seven rationales for maintaining the rejections, each quoted in full below. After each rationale/basis for maintaining each rejection is *quoted (and italicized), Applicant provides rebuttal evidence and arguments.*

1. *"Regarding Satoh the reference teaches a decellularized ECM sheet from a Urodele as claimed. The reference teaches an acellular ECM, or decellularized, which would inherently have the claimed retention of structural and functional integrity as claimed. Even if the ECM is crosslinked with glutaraldehyde, it would still retain sufficient structure and function to permit and support use of the matrix as a substrate for growth of cells in vivo or in vitro, as defined by the instant specification on page 12, thus inherently meeting the claimed limitations.* ***Absent clear evidence of a structural or functional difference between the claimed ECM and that of Satoh, the arguments are found unpersuasive."***

In response, Applicant has amended the claims (which implicitly excluded a glutaraldehyde cross-linking step and cross-linked graft) to include an explicit definition contained in the specification which necessarily excludes a glutaraldehyde cross-linking step and cross-linked graft. Applicant also will soon file a further Declaration of Dr. Ashley Seifert, to demonstrate that a product made by the Satoh method does not meet the limitations of the claims. Data will be presented to confirm clear structural and functional differences between the ECM prepared according to the Satoh process as compared to that which is presently claimed and described in the instant specification.

The product of claim 5 as herein amended (and claims 20 and 22 have been likewise amended) is prepared by a process in which the active affirmative step of "decellularizing" is

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characterized as producing "a decellularized ECM" As defined in the specification, this means reducing antigenicity to an extent where the matrix would be considered non-toxic as a xenograft. The newly cited reference makes clear that the glutaraldehyde treatment of Satoh will result in a product that "incites significant toxic, inflammatory, immune, and calcification responses", which is outside the scope of the claims.

Based on the clear structural and functional differences shown between the ECM prepared as disclosed and claimed in the present application, and the material produced according to the Satoh process, it is urged that the Applicant has met its burden of demonstrating the novelty of the claimed subject matter and has also limited the scope of the rejected claims to be commensurate with the scope of novelty by excluding from the scope of the rejected claims products produced in the prior art by a cross-linking step in the decellularization of the ECM material, whether that be a mammalian ECM, an amphibian ECM, or an Urodele or axolotl ECM. Reconsideration and withdrawal of this ground for rejection is therefore respectfully requested.

2. *"Regarding the argument that the prior art does not teach the claimed process steps, it is noted that* ***the claim fails to recite active process steps*** *as argued. Rather,* ***the claims only recite functional descriptive language,*** *without any specific step to meet the limitations. The claims do not require any step for removing cellular debris, any amount of ECM,* ***nor do they exclude crosslinking.*** *Thus, the argument is not commensurate in scope with the claimed invention."*

Applicants disagree, particularly in view of the claims as amended. The claims require decellularization to a degree where the ECM is sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix to an extent where the matrix would be considered non-toxic as a xenograft. The specification cites many methods for accomplishing this, which methods are known in the art. Satoh, however, does not employ such methods because Satoh employs a glutaraldehyde cross-linking step which prevents removal of cellular materials and also results in a toxic graft.

The Examiner is urged to keep in mind the *entire disclosure* and thrust of the present invention. Applicant discovered that if cellular material is removed from certain Urodel tissue, then the remaining native material is *immunoprivileged*. The application is based on that novel and

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unexpected discovery. The unequivocal thrust of the application is to eliminate the cells to yield that immunoprivileged material. Satoh not only does not eliminate the cells, because Satoh cross-links cellular organelles like nuclei in place, but Satoh also cross-links the material in a manner to destroy the immunoprivileged state in same manner as is described in the newly cited reference. Satoh's process is the antithesis of that described and presently claimed, as would be understood by one of ordinary skill in the art.

As stated in the application:

"Moreover, the cutaneous and subcutaneous structures of an axolotl resemble that of the amniotic/chorionic interface, in the sense that axolotl skin is composed of fused ectoderm and mesoderm. Axolotl skin also is rich in growth factors and antimicrobial peptides, similar to the AM. Furthermore, **axolotl ECM is immunoprivileged and** contains collagen III and tissue inhibitors of metalloproteinases (TIMPs), inter alia, also in resemblance to AM. **Of particular importance for transplantation purposes, more generally, is the immunologically privileged state of the human neonate (e.g., fetal dermis) and the AM, a state mirrored by axolotl ECM, whereby immogenicity is rarely manifested.**"

Accordingly, it is urged that the claims as amended should be sufficient to distinguish the Satoh process and product, which includes a glutaraldehyde cross-linking step, destroying immunoprivilege and retaining cellular components. Gluteraldehyde treatment interferes with Applicant's decellularization step. This will be further supported in a further Declaration of Dr. Ashley Seifert.

3. "Regarding applicant's assertion that the examiner has not proven the ECM of the prior art has eliminated antigenicity and is suitable for xenograft, it is maintained that the prior art teaches acellular collagen, acellular ECM, and decellularized basement membranes, which means the ECM ***are free of cellular components. As defined by applicant on page 12, being free of cells and cellular components eliminates or reduces antigenicity as well as makes the material suitable for xenograft.*** *Further, as the claimed invention fails to recite any specific step for making the graft, the claims neither require nor exclude any step in achieving such a decellularized ECM. As such, the claims read on the prior art.* ***It is noted that the burden is on applicant to provide arguments and evidence establishing that the claimed product is different from that of the prior art, and that such arguments and evidence must be commensurate in scope with the claimed invention.****"*

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Again, carrying out the invention is straight forward, once the initial unexpected discovery is made. Once it is known that the native cell-free material is immunoprivileged, then removing cells to produce a non-toxic graft is straight forward. It is black letter law that the office should take into account the unexpected discovery which forms the basis of the invention. Applicant believes it has met its burden to show its product is different from a glutaraldehyde treated product, and will submit a further Declaration to further support this view.

4. "Regarding the intended use of the compositions, it is noted that intended use of the claimed composition does not patentably distinguish the composition, per se, ***since such undisclosed use is inherent in the reference composition.*** *In order to be limiting, the intended use must create a structural difference between the claimed composition and the composition of the prior art. In the instant case, the intended use fails to create a structural difference, thus, the intended use is not limiting. Please note that when applicant claims a composition in terms of function, and the composition of the prior art appears to be the same, the Examiner may make rejections under both 35 U.S.C 102 and 103 (MPEP 2112).* ***Furthermore, the claimed process does not recite any active steps that must be practiced to arrive at a product with structural or physical differences. It is reiterated that the claims only recite functional descriptive language, without any specific step to meet the limitations. As such, the claims encompass any decellularized ECM with structural/functional integrity and has been decellularized. Thus, the argument is not commensurate in scope with the claimed invention****."*

As discussed above, as amended, the claims in the present application have been rendered commensurate in scope with a product produced by a process wherein treatment with an agent like glutaraldehyde is excluded. Applicant did not invent a new process of decellularization. Applicant invented a new graft material, based on the discovery that such material is immunoprivileged, as would be understood by one of ordinary skill in the art reading the instant specification. The claim scope, as amended, clearly does not read on the Satoh product.

5. Regarding the combination of Asem and Seifert, it is noted that it is the combination of references that is relied upon. Asem teaches decellularized ECM derived from amphibians in the form of sheets that can be adhered together (or laminated), powders (or particles) or reconstituted gels, which are made under alkaline conditions. Asem teaches the ECM

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compositions may be used as tissue grafts constructs, with cell culture, or culturing human cells wherein it is preferably sterilized prior to use. ***Seifert is relied upon to demonstrate that Urodele, specifically axolotl, was a known amphibian source from which a decellularized ECM may be obtained. Moreover, Seifert provides evidence of a functionally equivalent amphibian source of ECM that can be decellularized and used in the compositions of Asem with a reasonable expectation for success.***

With the prior response, the Expert Declaration of Dr. Ashley Seifert was provided in which he stated on the record his opinion that, as an expert in the field, and as principal author of the Seifert reference being cited in combination with the Asem reference, that he contests the Examiner's assertion that the Seifert reference teaches or suggests use of Urodele or axolotl as a functionally equivalent amphibian ECM to the material used in Asem. Dr Seifert opined that one skilled in the art reading Asem and Seifert together would not have had a reasonable expectation for successfully obtaining a functional decellularized ECM.

First, there is no teaching in Asem (or in Seifert et al.) that there is any tissue (other than placental tissue) that is the "functional equivalent" to follicular ECM. By contrast, as noted in the Declaration of Dr. Seifert, Asem teaches, if anything, that follicular tissue, like placental tissue, is unique.

Dr. Seifert's expert testimony is directly contradictory to the Examiner's assertion that "***Seifert is relied upon to demonstrate that Urodele, specifically axolotl, was a known amphibian source from which a decellularized ECM may be obtained."*** The Examiner has failed to show that Seifert may properly be relied on for the teaching asserted by the Examiner. The Examiner has failed to show how or where Seifert provides such evidence. It is also directly contradictory to what Dr. Seifert, a person skilled in the art and the author of the Seifert reference, has stated. Seifert is directed to studying wound healing in the axolotl as a model for wound healing in mammals. Any "ECM" material produced is a cross-linked material, much as is the Satoh material, produced for the purpose of analysis, and is excluded from the scope of the present claims as amended and the newly introduced claims.

6. Regarding a teaching away, it is acknowledged that Seifert teaches different outcomes than applicant. However a different outcome is not a teaching away from functionality

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of the ECM. Seifert is not cited to motivate one in the art to use a crosslinked ECM from Urodele in the compositions of Asem, but rather ***to evidence it was a known source of amphibian ECM that can be decellularized and used as the amphibian ECM in the compositions of Asem with a reasonable expectation for success.*** *Please note that the instant claims do not exclude or require any step for producing the decellularized ECM, nor do they require or exclude any particular type or source of ECM other than being any tissue from a Urodele. Thus, the arguments are not commensurate in scope with the claimed invention."*

The Applicant and the author of the Seifert reference itself contests the Examiner's assertion that Seifert may properly be used "***to evidence it was a known source of amphibian ECM that can be decellularized and used as the amphibian ECM in the compositions of Asem with a reasonable expectation for success".*** Asem teaches, if anything, that follicular tissue, like placental tissue, is unique. The Examiner has not shown anything to the contrary. The Examiner has not shown or provided a reasoned basis for why someone would ever substitute the material of Seifert for that of Asem with an expectation of success. There has been no showing on the record of any teaching or suggestion in the Seifert reference of the production of decellularized ECM as claimed. The entire reference is directed to the study of ECM and its role *in vivo* in wound healing. It is only through hindsight extrapolation from the present disclosure that the Examiner boot-straps the Seifert reference, over the objection of the Author himself, into a rejection of the present claims. Further, just as in Satoh and in Seifert, Asem provides for a cross-linked product, which is excluded from the present claims.

7. "In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. See In re McLaughlin, 443 F.2d 1392, 170USPQ 209 (CCPA 1971)."

Simply stating that "*obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning",* the Examiner does not demonstrate the permissibility of the hindsight at

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play in the present situation. Here, all of the cited and relied upon art relates to cross-linked products and processes using cross-linking. This is explicitly excluded from the pending claim set as amended, and from the newly introduced claim set. In addition, in this instance, the Examiner ignores the main teaching in the Asem reference that the tissue used by Asem is unique and ignores the declaration by the Author of Seifert as to the meaning of the Seifert reference.

Reconsideration and withdrawal of this ground for rejection is respectfully requested.

SUMMARY:

All grounds for objection or rejection of claims asserted in the outstanding Office Action have been addressed and overcome herein by Amendment of the claims and by the remarks, arguments, and evidence, including that provided by Dr. Ashley Seifert. The objections/rejections under 35 USC 112 have been addressed by claim amendments. The rejection of claims under 35 USC 101 as being drawn to ineligible subject matter has been withdrawn. Likewise, it is urged, the rejection of claims under 35 USC 102 and 103 have been overcome by claim amendments and by the arguments and evidence provided herein and in the Declaration of Dr. Seifert.

It is respectfully urged that all claims pending in this application are in condition for allowance.

In the event a telephonic or in-person interview would assist in bringing prosecution in this application to a successful conclusion, the Examiner is invited to contact the undersigned to arrange such an interview.

Dated: October 22, 2018

Respectfully submitted,

By: /Edward R. Gates/
Edward R. Gates
Registration No.: 31,616
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

CLEAN LISTING OF ALL CLAIMS PENDING (ALBEIT SOME ARE WITHDRAWN BUT NOT CANCELED) IN THIS APPLICATION:

1. A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

6. An isolated, decellularized Urodele ECM.

7. The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. The isolated ECM of claim 6, wherein the ECM includes basement membrane.

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9. The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. The isolated ECM of claim 6, wherein the ECM is a sheet.

12. The isolated ECM of claim 11, wherein the sheet includes perforations.

13. The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

16. A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or

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a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by

removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and sterilizing said tissue.

21. The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and

sterilizing said tissue.

23. A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. The tissue culture system of claim 22, wherein the cells are human cells.

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25. Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. The method of claim 30, further comprising reconstituting the powder as a gel.

32. The method of claim 29, further comprising sterilizing the decellularized ECM.

33. The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

36. The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

38. The tissue graft of claim 5, wherein the Urodele is an Axolotl.

40. The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

43. The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

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46. The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel, a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. The tissue graft of claim 49, wherein the ECM is a powder.

51. The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. The tissue graft of claim 49, wherein the ECM is a paste.

54. The tissue graft of claim 49, wherein the ECM is a gel.

55. The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. The tissue graft of claim 49, wherein the ECM is a mesh.

57. A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial

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ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. The tissue graft of claim 57, wherein the agent is a growth factor.

60. The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

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62. The tissue graft according to claim 5 wherein said
(ii) removing sufficient cellular components of the sample, is carried out without cross-linking, to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft.

63. The tissue graft according to claim 5 which is not cross-linked.

64. The sterile medical implant according to claim 20 wherein said decellularization is carried out without cross-linking.

65. The material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM according to claim 22 produced without cross-linking.

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IN THE CLAIMS:

Please replace all prior listings of claims in this application with the following listing of claims.

A clean set of claims, as herein amended, is provided as an attachment at the conclusion of this paper.

1. (Withdrawn) A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. (Withdrawn) The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. (Withdrawn) The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. (Withdrawn) The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. (Currently amended) A tissue graft comprising extracellular matrix components derived from a Urodele, said tissue graft derived by:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix; and

b. decellularizing said tissue sample <u>to produce decellularized extracellular matrix</u> by ~~a process comprising (i) retaining the structural and functional integrity of said tissue sample; and~~

~~(ii)~~ removing sufficient cellular components of the sample to reduce or eliminate

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antigenicity of the biomaterial making it suitable for use as a xenograft.

6. (Withdrawn) An isolated, decellularized Urodele ECM.

7. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. (Withdrawn) The isolated ECM of claim 6, wherein the ECM includes basement membrane.

9. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. (Withdrawn) The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a sheet.

12. (Withdrawn) The isolated ECM of claim 11, wherein the sheet includes perforations.

13. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. (Withdrawn) The isolated ECM of any one of claims 6-13, therein the ECM is

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sterile.

16. (Withdrawn) A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. (Currently amended) A sterile medical implant comprising sterile, isolated, decellularized Urodele Extracellular Matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing and sterilizing said tissue sample to produce decellularized extracellular matrix by ~~a process which comprises:~~

~~(i) retaining the structural and functional integrity of said tissue sample; and (ii)~~ removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and ~~(iii)~~ sterilizing said tissue.

21. (Original) The sterile medical implant of claim 20, wherein the implant is a

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biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Currently amended) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM wherein said isolated, decellularized Urodele ECM is produced by a process comprising:

a. obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix;

b. decellularizing said tissue sample to produce decellularized extracellular matrix by ~~a process which comprises: (i) retaining the structural and functional integrity of said tissue sample; and (ii)~~ removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft; and ~~(iii)~~ sterilizing said tissue.

23. (Withdrawn) A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. (Withdrawn) The tissue culture system of claim 22, wherein the cells are human cells.

25. (Withdrawn) Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. (Original) A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. (Withdrawn) A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. (Withdrawn) A method of preparing a biologic material comprising (A)

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obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. (Withdrawn) The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. (Withdrawn) The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. (Withdrawn) The method of claim 30, further comprising reconstituting the powder as a gel.

32. (Withdrawn) The method of claim 29, further comprising sterilizing the decellularized ECM.

33. (Withdrawn) The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

34-35. (Canceled)

36. (Previously presented) The tissue graft of claim 5, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (Previously presented) The tissue graft of claim 36, wherein said process further comprises subjecting said sample to sterilization.

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38. (Previously presented) The tissue graft of claim 5, wherein the Urodele is an Axolotl.

39. (Canceled)

40. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM includes basement membrane.

41. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (Currently amended) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug ~~or an agent found in a human, whether isolated, synthetically or recombinantly produced~~.

43. (Previously presented) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

45. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is in the form of Urodele ECM particles.

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46. (Previously presented) The sterile medical implant of claim 20, wherein the Urodele ECM is a reconstituted gel.

47. (Previously presented) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (Previously presented) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

49. (Previously presented) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a powder, a paste, a gel, a sheet, or a mesh, and wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM.

50. (Previously presented) The tissue graft of claim 49, wherein the ECM is a powder.

51. (Previously presented) The tissue graft of claim 50, wherein the powder has a particle size between 125 and 850 microns.

52. (Previously presented) The tissue graft of claim 50, wherein the powder has a particle size of less than 250 microns.

53. (Previously presented) The tissue graft of claim 49, wherein the ECM is a paste.

54. (Previously presented) The tissue graft of claim 49, wherein the ECM is a gel.

55. (Previously presented) The tissue graft of claim 54, wherein the gel comprises a synthetic polymer.

56. (Previously presented) The tissue graft of claim 49, wherein the ECM is a mesh.

57. (Previously presented) A tissue graft according to claim 5 comprising sterile, isolated, decellularized Urodele extra cellular matrix (ECM), wherein the ECM is a sheet, wherein the ECM is parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug.

58. (Previously presented) The tissue graft of claim 57, wherein the agent is an anti-microbial agent.

59. (Previously presented) The tissue graft of claim 57, wherein the agent is a growth factor.

60. (Previously presented) The tissue graft of claim 57, wherein the sheet is at least two sheets of isolated Urodele decellularized ECM laminated to one another.

61. (Previously presented) The tissue graft according to claim 49 wherein the Urodele ECM is Axolotl ECM and is in a form selected from the group consisting of: a powder; a paste; a gel; a mesh; a sheet of parietal ECM, mesothelial ECM, thoracic cavity ECM, abdominal cavity ECM, pericardium ECM or dermal tissue ECM, and wherein the sheet of ECM is infused with, coated with, or attached to an agent xenogeneic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, or a drug; and at least two sheets of isolated Urodele decellularized ECM laminated to one another.

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62. (New) The tissue graft according to claim 5 wherein said

(ii) decellularization is carried out without cross-linking .

63. (New) The tissue graft according to claim 5 wherein said decellularization is carried out without cross-linking.

64. (New) The sterile medical implant according to claim 20 wherein said decellularization is carried out without cross-linking.

65. (New) The material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM according to claim 22 produced without cross-linking.

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DOCKET NO.: S1860.70000US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	R. A. Davis
Art Unit:	1651

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: October 22, 2018 Electronic Signature for: /Eileen M. MacKenzie/

MAIL STOP RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO ACCOMPANY A REQUEST FOR CONTINUED EXAMINATION ("RCE")

The claims pending in this application are Claims 1-33, 36-38, and 40-61; Claims 1-4, 6-19, 23-25, and 27-33, stand withdrawn from consideration; Claims 34-35 are cancelled herein below; leaving claims 5, 20-22, 26, 36-38 and 40-61 under examination. Per the Final Office Action mailed in the subject application on 04/20/2018, all claims under consideration stand rejected under 35 U.S.C. §§ 102 and 103. Claims 5, 20, 21, and 42 are amended herein below and new claims 62-65 are introduced, leaving claims 5, 20-22, 26, 36-38, and 40-65 under examination. The Independent Claims are Claims 5, 20, 22, and 26 (four independent Claims).

Amendments to the claims begin on page 2 of this paper, and Remarks and Arguments in support of the patentability of the claims, as herein amended, begin on page 10 herein below.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: ISE Professional Testing & Consulting Services, : Inc., and Inventor Ryanne Early
TTILE OF THE INVENTION	: Decellularized Biomaterial From Non-Mammalian : Tissue
SERIAL NUMBER	: 14/759,895
FILING DATE	: PCT Filed Jan. 9, 2014; 371 date: Jul. 8, 2015 : Priority Date Jan. 9, 2013 Prov. 61/750,555
EXAMINER	: Davis, Ruth A.
GROUP ART UNIT	: 1651
ATTNY. DOCKET NO.	: S1860.70000US01
CONFIRMATION NO.	: 3358

EXPERT DECLARATION UNDER 37 C.F.R. 1.132
TO ACCOMPANY THE FILING OF A REQUEST FOR CONTINUED EXAMINATION IN THE SUBJECT APPLICATION

THE COMMISSIONER OF PATENTS & EXAMINER DAVIS:

1. I hereby declare that I am the same Ashley W. Seifert who has previously executed an Expert Declaration, on 02-15-2018, and which has been previously filed in support of patentability of the subject matter claimed in the subject patent application.

2. I am aware that, nonetheless, in an Office Action dated 04/20/2018, the Examiner has maintained the rejection of certain claims as lacking novelty, in light of Satoh et a1. (2012)[1], and as being obvious in light of Seifert in combination with Asem. I am also aware of, have read, and I concur with the arguments and claim amendments that are being filed concurrently with this Declaration, in support of the novelty and non-obviousness of the pending claims, notwithstanding citation of the Satoh reference, and the Seifert/Asem combination of references.

NOVELTY OVER SATOH:

3. In my previously-filed Declaration, I expressed my expert opinion that, not only are the

[1] Satoh, A., Hirata, A. & Makanae, A. Collagen reconstitution is inversely correlated with induction of limb regeneration in Ambystoma mexicanum. *Zoological science* **29**, 191-197 (2012).

processes disclosed and used by Satoh, the "Satoh Process", to produce a product, the "Satoh Product", different from the "Current Process" used to produce "Current Product" as disclosed in the subject patent application, but that, per force, because of the differences in the processes of production between the Satoh Process and the Current Process, the products produced, namely the Satoh Product and the Current Product, would be different from each other in a number of significant respects. The Examiner appears to be requesting objective evidence to support the novelty and non-obviousness of the claims drawn to the current Product.

4. In what follows, I provide direct experimental evidence that the opinion that I provided previously is in fact correct: The Current Product is significantly different from the Satoh Product in ways that are significant to the claims and patentability and use of the Current Product as a xenograft with low antigenic load as compared to the Satoh process, which, at least by virtue of cross linking, is different and it carries significant residues of cellular structures and intact cells, such that it does not meet the claim limitations.

5. Product Produced Using the Satoh method to prepare a product from axolotl skin, "the Satoh Product".

In what follows, use of the term "I" includes work conduced directly by myself or work conducted by others under my direction; for brevity, all such work reported below and conclusions drawn are simply referenced as "I", as actions taken by me, or as my conclusions drawn by me, after reviewing the results of the experiments set forth in detail below.

a. Conclusion:

While the Satoh Process results in substantial removal of the epidermis from a piece of native axolotl skin, it does not remove all the cells from the tissue, and the resultant Satoh Product also retains gland fragments in the dermis.

By contrast, the product produced by the inventor according to the subject patent application, likewise starting with a piece of native axolotl skin, results in a product, the "Current Product",

which is completely devoid of cells from the tissue, there is no retention of gland fragments, and it is therefore, in my opinion, new and substantially different from the Satoh Product.

b. Summary of Experiment and Conclusions:

Experiments were undertaken by me (or under my direction) to determine if use of the procedure disclosed in Satoh et al., (2012), "the Satoh Process", to process a piece of native axolotl skin results in production of a product, "the Satoh Product", that is devoid of cells. On examination of the Satoh Product, I found that:

i. The Satoh Process resulted in substantial removal of the epidermal layer of the native tissue, but the Satoh Product retained cells throughout the dermis, see Figure 1 below.

ii. Cells were visible with a simple H&E stain, as compared to the Current Product, as shown in Figure 5 of the published patent application, US2015/0352257, which shows H&E staining of the Current Product, showing no retention of cells.

iii. Histological analysis also revealed the retention of gland fragments in the dermis, see Figure 1, again, as compared to, e.g. the Current Product, see Figure 5 of the published patent application, US2015/0352257, which shows that the Current Process results in disruption of the glands of the dermis, along with the all cellular constituents.

iv. Using the Hoechst nuclear stain, which binds to DNA, cells can be seen throughout the dermis of the Satoh Product (Figure 1). In contrast, the Current Product contains, on examination, no cells or evidence of cellular debris in axolotl skin (Figure 2).

v. In order to control for possible differences caused by normal fixation, we also analyzed unfixed salamander skin recovered from the Satoh process (Figure 1).

c. Methods:

i. Sample Collection and Decellularization

Following Satoh et al. (2012), an 8x6mm^2 biopsy was harvested from albino *Ambystoma mexicanum* dorsal skin (n=3) and immediately placed in PBS containing 0.05% EDTA. Samples were incubated and agitated at room temperature for 4 hours. Each sample was then rinsed with distilled H_2O for 5 minutes. A solution of distilled H_2O, 0.01% Tween-20, 1.74ug/mL PMSF, and 20.18ug/mL glutaraldehyde was prepared and added to each tube (5mL solution per sample). Samples were incubated and agitated for 48 hours at room temperature. After incubation, samples were rinsed in distilled H_2O for 5 minutes. A DNase solution was prepared according to the protocol from Promega and the samples were incubated and agitated at 37°C for 1 hour. The DNase stopper solution was added and tissue was incubated for 10 minutes at 60°C. Samples were then rinsed with distilled H_2O for 5 minutes. Each sample was then cut in half using a razor blade, one half being dehydrated in 70% ethanol (3x10min) and prepared for histological-processing, and the other was placed into 10% neutral buffered formalin overnight at 4°C. The fixed samples were then rinsed with distilled water for 3x10 minutes each and 70% ethanol for 3x10 minutes each. Samples were then embedded in paraffin using a Histos5 processor, cut at 5um thickness, and stained using standard hematoxylin and eosin (H&E) protocol (see below). A second set of sections were deparaffinized, stained using Hoechst nuclear stain at a concentration of 1:10,000 for 5 minutes, and mounted with Prolong Gold (Life Technologies).

ii. Hematoxylin and Eosin Staining

Slides containing three samples each were deparaffinized by heating at 37°C for 10 minutes, submerging in 2x xylenes for 5 minutes each, 2x 100% ethanol for 5 minutes each, 95% ethanol for 2 minutes, 70% ethanol for 3 minutes, and 2x distilled water for 1 minute each. Samples were then stained in modified Mayers hematoxylin (American Mastertech) for 8 minutes, rinsed in running tap water for 30 minutes to darken the stain, dunked in 95% ethanol 10x, stained in eosin Y/phloxine B (American Mastertech) for 45 seconds, and cleared in 95% ethanol for 2 minutes.

Lastly, samples were dehydrated in 100% ethanol 2x for 2 minutes each and cleared in 3x xylenes for 2 minutes each. Samples were mounted wet using Cytoseal XYL (Thermo Fisher).

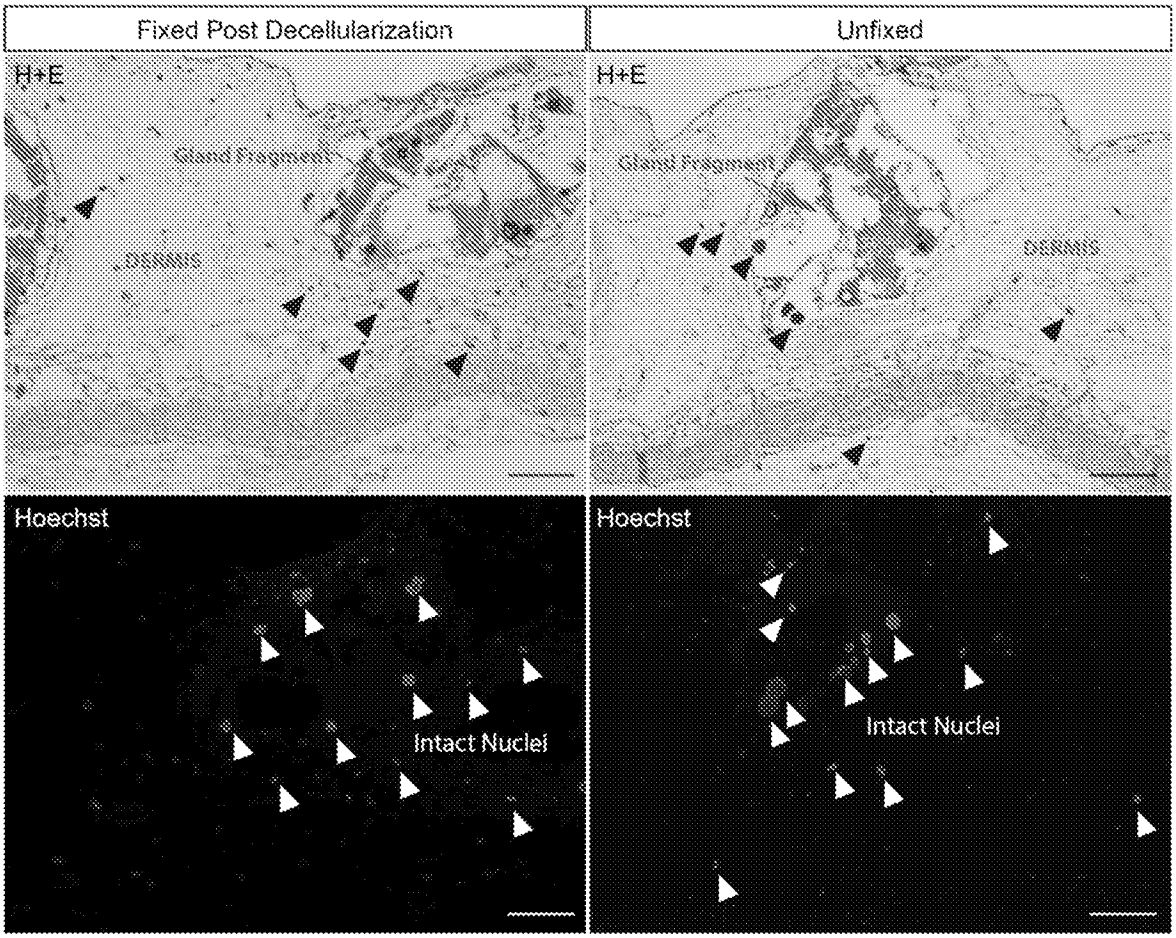


Figure 1. *Ambystoma mexicanum* tissue decellularized according to a procedure published by Satoh et al., 2012 and stained with hematoxylin/eosin and Hoechst nuclear stain. Multiple nuclear bodies can be seen in the washed tissue samples of both the fixed and unfixed dorsal skin sections (black and blue arrows), including fragments of axolotl dermal glands. Intact nuclei are clearly visible within the glands and throughout the dermis. Scale = 100um.

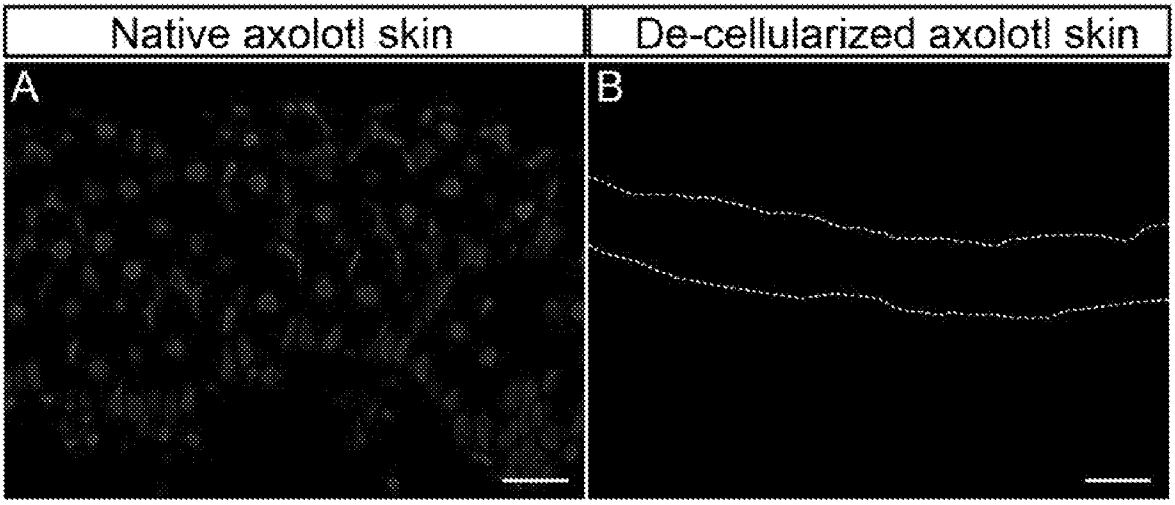


Figure 2. NEOMATRIX®, axolotl skin tissue (an 8x6mm^2 biopsy harvested from albino *Ambystoma mexicanum* dorsal skin) and decellularized without cross-linking by the inventor in the subject patent application, reveals a product in which all cells are gone following de-cellularization. (A) Native axolotl skin clearly shows the presence of cell nuclei stained with Hoechst Nuclear Stain (blue stain). (B) In contrast, cell nuclei are not visible in NEOMATRIX® product. White dotted lines show the upper and lower boundaries of the NEOMATRIX® tissue sample. Scale bars = 50µm.

6. In contrast to the Satoh Product, the Current Product, produced according to the Current Process of the present invention, pursuant to the patent application, is produced by the inventor by a process consisting essentially of decellularizing the native tissue sample to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to render it suitable for use as a xenograft while retaining structural and functional integrity of the natural, cellular, tissue. This language should be read to exclude a process in which cross-linking is a component or in which cross-linking materials are employed. As discussed and shown experimentally, the product and process of producing the product disclosed in Satoh is neither the same as or even similar to the product and process of producing the product pursuant to the claims, as currently amended, in the subject application.

NON-OBVIOUSNESS, NOTWITHSTANDING SEIFERT IN COMBINATION WITH ASEM

7. I am further aware that the Examiner is maintaining the rejection of certain pending Claims under 35 U.S.C. 103 as being unpatentable over Asem et al. (US 6485969) in view of Seifert et al. (2012); that Asem is being cited as providing a teaching of decellularized ECM comprising basement membranes (col.1 line 66 - col.2 line 1-18, col. 5 line 23-39), derived from amphibians (col. 4, line 35-40); that the Asem ECM is said to be in the form of sheets that are adhered together (or laminated) (col.6 line 20-32), powders (or particles) or reconstituted gels, made under alkaline conditions (col, 6 line 43-64); that the ECM compositions may be used as tissue grafts constructs (col. 4 line 30-35) or with cell culture comprising growth factors for culturing human (co1. 11 line 40-45) cells (or are infused with, coated with, or attached to a growth factor) (col. 7-8) or as a coating wherein it is preferably sterilized prior to use (col.7 line 30-45). I further understand that the Examiner has conceded that "Asem does not teach the ECM wherein the amphibian is a Urodele, or axolotl." However, that notwithstanding, I further understand that the Examiner is asserting that "at the time the instant claims were filed, in following the teachings of Asem, one of ordinary skill in the art would have been motivated to use any known functionally equivalent amphibian ECM in the compositions of Asem with a reasonable expectation for successfully obtaining a decellularized ECM. At the time the instant claims were filed, axolotls were known amphibian sources of ECM and components derived therefrom. In support, Seifert teaches axolotl ECM and its basement membrane are useful for regenerative therapies and wound repair (abstract) as well as blueprints for healing in vertebrates (entire document). As such, at the time the claims were filed, it would have been obvious to one of ordinary skill in the art to use ECM from axolotl in the compositions of Asem, since it was a known, functionally equivalent amphibian ECM and because of its known benefit in cellular regeneration. Thus, the claims are rendered obvious."

8. I have previously provided my expert opinion, and as principal author in the Seifert publication which the Examiner is citing, in combination with the Asem reference, that the combination proposed by the Examiner in rejecting the claims does not render the claimed subject matter unpatentable for obviousness: That the Seifert et al. reference, if anything, teaches away from the combination with Asem proposed by the Examiner and that there is no basis provided in the Seifert publication to support the proposition that the Seifert reference provides "evidence that it would have been obvious to use Urodele or axolotl as a functionally equivalent amphibian ECM

with a reasonable expectation for successfully obtaining a functional decellularized ECM." The Examiner has not provided any citation to any aspect of the Seifert publication which would support this allegation. As shown above in relation to the Satoh Product and the Satoh Process, ECM produced according to Asem, even if made from Urodele or even axolotl follicular tissue, would be a different product than if that same follicular tissue were obtained from Urodele or axolotl follicular tissue and were produced by treatment according to the process of the present disclosure and claims. I further opined that it cannot validly be stated that Seifert's histological section preparation steps, carried out to produce tissue samples adapted for analysis of the scar free tissue regeneration process that occurs *in vivo* in the axolotl, wherein formalin fixing axolotl tissue specimens at different stages of *in vivo* wound healing, is adequate to teach or suggests a process used to prepare a tissue product, or the tissue product produced thereby, which is not formalin fixed, is decellularized, and thereby made suitable for implantation into another species than that from which it was derived. I understand that the claims have been amended to explicitly exclude cross-linking during decellularization and that a cross-linked product is excluded from the scope of the claims and on that basis alone, the claims should be found to be directed to novel and non-obvious subject matter.

9. In my opinion, much as with the Satoh Product produced according to the Satoh Process, as described above, the Seifert material fails to meet the present claims' requirements of retaining structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial to make it suitable for use as a xenograft while avoiding cross-linking, and I further note that in Asem, it is disclosed at column 8, lines 4-14, that:

"The present basal lamina compositions may be sterilized using conventional sterilization techniques including tanning with glutaraldehyde, formaldehyde tanning at acidic pH, ethylene oxide treatment, propylene oxide treatment, gas plasma sterilization, gamma radiation, and peracetic acid sterilization. A sterilization technique which does not significantly weaken the mechanical strength and biotropic properties of the material is preferably used. For instance, it is believed that strong gamma radiation may cause loss of strength in the basal lamina."

Accordingly, Asem, like Satoh and Seifert, teaches a cross-linked material, while the present claims exclude cross-linking during decellularization and cross-linked material. Those skilled in the art aware of Asem's teaching, had they turned to or become aware of the Seifert reference, at most, may have thereby considered the possibility of acquiring ECM produced in response to injury, rather than, as in the subject application, using native, uninjured ECM_{ev}, which does not contain the same components as the ECM produced during healing *in vivo* as defined in detail in the Seifert publication. In any event, the process of producing such a material from each of these references includes a cross-linking material, which the presently amended claims exclude.

10. In the attached publication, Toxicol. Int. 2012, Jan.-Apr.; 19(1):51-58, (see https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3339246/?report=printable), entitled:

"Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium", Umashankar et al., report that (bolded emphasis added):

"Glutaraldehyde-stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification is reported on account of glutaraldehyde treatment. In this study, commercially available glutaraldehyde cross-linked bovine pericardium was evaluated for its in vitro cytotoxic effect, macrophage activation, and in vivo toxic response in comparison to decellularized bovine pericardium. Glutaraldehyde-treated bovine pericardium and its extract were observed to be cytotoxic and it also caused significant inflammatory cytokine release from activated macrophages. Significant antibody response, calcification response, necrotic, and inflammatory response were noticed in glutaraldehyde-treated bovine pericardium in comparison to decellularized bovine pericardium in a rat subcutaneous implantation model. Glutaraldehyde-treated bovine pericardium also failed in acute systemic toxicity testing and intracutaneous irritation testing as per ISO 10993. With respect to healing and implant remodeling, total lack of host tissue incorporation and angiogenesis was noticed in glutaraldehyde-treated bovine pericardium compared to excellent host fibroblast incorporation and angiogenesis within the implant in decellularized bovine pericardium. In conclusion, using in vitro and in vivo techniques, this study has demonstrated that glutaraldehyde-treated bovine pericardium elicits toxic response compared to decellularized bovine pericardium which is not congenial for long-term implant performance."

11. Accordingly, the novel, immunopriviledged, non-cross linked, decellularized material according to this invention provides, in my opinion, a significant alternative to existing products, including mammalian and non-mammalian cross-linked ECM based products, and the Current Product produced by the Current Process meets the requirement in the claims of being non-cross linked and/or being decellularized by a process consisting essentially of:
(i) retaining the structural and functional integrity of said tissue sample; and
(ii) removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial making it suitable for use as a xenograft. The same cannot be said for the Satoh product, the Seifert Product, the Asem Product, their related processes, or any reasonable combination of the teachings in those references.

12. The signatory, being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements and the like may jeopardize the validity of the application or submission or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.



Ashley W. Seifert

10-17-2018
Date

Toxicol Int. 2012 Jan-Apr; 19(1): 51–58.
doi: 10.4103/0971-6580.94513
PMCID: PMC3339246
PMID: 22736904

Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium

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Abstract

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Glutaraldehyde-stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification is reported on account of glutaraldehyde treatment. In this study, commercially available glutaraldehyde cross-linked bovine pericardium was evaluated for its *in vitro* cytotoxic effect, macrophage activation, and *in vivo* toxic response in comparison to decellularized bovine pericardium. Glutaraldehyde-treated bovine pericardium and its extract were observed to be cytotoxic and it also caused significant inflammatory cytokine release from activated macrophages. Significant antibody response, calcification response, necrotic, and inflammatory response were noticed in glutaraldehyde-treated bovine pericardium in comparison to decellularized bovine pericardium in a rat subcutaneous implantation model. Glutaraldehyde-treated bovine pericardium also failed in acute systemic toxicity testing and intracutaneous irritation testing as per ISO 10993. With respect to healing and implant remodeling, total lack of host tissue incorporation and angiogenesis was noticed in glutaraldehyde-treated bovine pericardium compared to excellent host fibroblast incorporation and angiogenesis within the implant in decellularized bovine pericardium. In conclusion, using *in vitro* and *in vivo* techniques, this study has demonstrated that glutaraldehyde-treated bovine pericardium elicits toxic response compared to decellularized bovine pericardium which is not congenial for long-term implant performance.

Keywords: Calcification, decellularized bovine pericardium, glutaraldehyde-treated bovine pericardium, immunogenicity, toxic response, tissue incorporation

INTRODUCTION

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Glutaraldehyde-stabilized bovine pericardium is commercially available and used for medical application in the form of patches and bioprosthetic valves on account of its low immunogenicity and excellent durability.[1] Glutaraldehyde treatment produces stable cross-links in cellular and extra-cellular matrix proteins which substantially reduced graft immunogenicity.[2] However, it was also shown that such tissue had altered mechanical property, early mechanical failure, cytotoxicity, and incomplete suppression of immunological recognition.[2–4] Besides this severe calcification was noticed in glutaraldehyde-treated

bovine pericardium in pediatric patients.[4] An emerging alternative to glutaraldehyde treatment for reducing xenograft immunogenicity is decellularization.[5,6] Use of decellularized xenograft is an approach where acellular tissue matrices are produced by selective removal of cellular components that are believed to promote immunogenicity and calcification. These acellular matrices promote induced regeneration through remodeling of the prosthesis by neo-vascularization, re-cellularization, and laying of new extracellular matrix by the host. The remodeled decellularized xenografts may functionally and structurally integrate into the host tissue.[6]

In this study, the *in vitro* and *in vivo* toxic effects of glutaraldehyde-treated bovine pericardium in comparison to decellularized bovine pericardium are reported. The *in vivo* effects of this toxic response such as inflammatory potential, immunogenicity, calcification potential, and lack of tissue incorporation are discussed as this may have effect on the long-term implant performance in clinical situation.

MATERIALS AND METHODS

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Glutaraldehyde-treated bovine pericardium

Commercially available glutaraldehyde-treated bovine pericardium (Glut BP) approved for clinical use in India was used in this study. This treatment group was named as Glut BP.

Decellularized bovine pericardium

Decellularization of bovine pericardium (Dcl BP) was achieved using a proprietary process based on a nondetergent method. Decellularization is confirmed by demonstrating the absence of nuclear remnants using routine HE staining as well as by the use of specific nuclear stain such as Hoechst 33258 on 5 μm paraffin embedded sections (n = 10). Besides this, residual DNA was extracted from 100 mg decellularized BP using DNA-XPress Kit (Hi-Media) and concentration was estimated spectrophotometrically (n = 3). This was followed by demonstration of extractable DNA by 1% agarose gel electrophoresis. The structural content was evaluated using histochemical staining of 5 μm paraffin embedded sections using Movats Pentachrome staining (n = 10). Decellularized bovine pericardium was sterilized by a proprietary process, and sterility was confirmed by routine sterility testing.

Collagenase susceptibility

This study was conducted to assess the ability of the graft to undergo *in vivo* degradation which will pave the way for laying the host extracellular matrix. In this test, the susceptibility of the test material to Collagenase II activity (from *Clostridium histolyticum*, 125 μm/mg, Sigma Aldrich) was studied. Approximately 50 mg (wet weight in triplicates) of glutaraldehyde-treated bovine pericardium (Glut BP) and decellularized bovine pericardium (Dcl BP) were weighed. To the weighed samples, 0.5 mL of 0.1 M Tris chloride (pH 7.4) containing 0.005 M calcium chloride and 0.05 mg/mL sodium azide was added. The samples were incubated at 37°C for 1 h with constant shaking. Collagenase enzyme was prepared in 0.1 M Tris chloride (pH 7.4) at 37°C and added in to the vials so as to make the final concentration to 2 U/mg of tissue. The vials were incubated at 37°C for 24 h, 72 h, and 7 days, at the end of which, the samples were centrifuged at 12,000 rpm for 20 min at 4°C and the remaining tissue samples are blotted in filter papers for 5 min to dry them. They were weighed using Afcoset Electronic Weighing Balance ER120A. The weight loss was determined by paired comparison before and after treatments.

In vitro response

xi toxic responses of Glut BP and Dcl BP were assessed by cytotoxicity studies using L929 fibroblast cells by a direct contact method as well as by studying the effect of the extract on L929 fibroblast metabolism by MTT assay. *In vitro* inflammatory response was studied by macrophage activation studies.

Cytotoxicity testing: a direct contact method

Cytotoxic potential of Glut BP and Dcl BP groups on account of their direct contact or due to leachables was evaluated by a direct contact method (ISO 10993-5). The test samples were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (high density poly ethylene, USP), and positive control (copper) in triplicate were placed on a subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at 37 ± 2°C for 24 ± 1 h, the cell culture was examined microscopically for cellular response around the samples. Cellular responses were expressed as noncytotoxic, mildly cytotoxic, moderately cytotoxic, and severely cytotoxic.

Cytotoxicity testing on extract

In vitro MTT assay on extract of Glut BP and Dcl BP in physiological saline was performed to measure the effect of extract on the metabolic activity of L929 fibroblast cells. The ability of the fibroblast cells to metabolize yellow-colored tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple-colored formazan was measured as an indication of its activity. Different dilutions (50% and 25%) of extracts of Glut BP sample, Dcl BP, positive control (dilute phenol), negative control (high density poly ethylene (USP)), and reagent control in triplicate were placed on the subconfluent monolayer of L929 cells. After incubation of cells with the extract at 37 ± 2°C for 2 h, extract and control medium were replaced with 50 μL MTT solution (1 mg/mL in the medium without supplements) wrapped with aluminum foils and were incubated at 37 ± 2°C for 2 h. After discarding the MTT solution, 100 μL of isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at 540 nm using a microplate reader. The data obtained were compared with the negative control.

Macrophage activation

Macrophage activation was studied by estimating inflammatory cytokine released by adhered macrophages on contact with materials belonging to different groups. THP-1 (human acute monocytic leukemia cell lines) cells were grown in RPMI 1640 (Sigma) containing 10 % v/v fetal bovine serum (Gibco), 160 U/mL benzylpenicillin, and 100 U/mL streptomycin (Sigma). The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 at 90% relative humidity. The differentiation into macrophages was induced by treating THP-1 cells in a 24-well plate for 24 h with RPMI-1640 containing 20 ng/mL phorbol 12-myristate 13- acetate (PMA). Thereafter, a fresh medium was added and cells were grown for another 24 h under similar conditions. Release of proinflammatory cytokines (IL-1b, IL-6, and TNF-a) from THP 1 cells upon contact with triplicate samples of Glut BP and Dcl BP and positive control (bacterial lipopolysaccharide) at 20 pg were carried using ELISA. The concentration of cytokines in the culture supernatant was quantified by a specific ELISA kit (U-CyTech biosciences, Netherlands) as per manufacturer instructions.

In vivo response

In vivo toxic response was studied by conducting an acute systemic toxicity testing (ISO 10993-11) and an intracutaneous (intradermal) reactivity test (ISO 10993-10). *In vivo* response such as immune response, calcification response, and tissue response was studied by juvenile rat subcutaneous implantation for 60

days. The animal test protocols were approved by Institutional Animal Ethics Committee and animal care and maintenance were done as per CPCSEA guidelines.

Acute systemic toxicity testing (ISO 10993-11)

This test was done to evaluate the systemic response of mice following intraperitoneal and intravenous injection of cotton seed oil and physiological saline extract of the test samples, respectively. The test samples were prepared by washing the samples in sterile normal saline for three wash cycles, with each cycle lasting for 10 min. The samples were dried at 37°C for 24 h. Two grams each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and cotton seed oil for 72 h at 50°C. Then, 50 mL/kg body weight of the above extracts were injected through intravenous (n = 5) and intraperitoneal routes (n = 5), respectively, in separate groups of randomly selected health active mice. Injection of plain extraction media in another set of animals served as control. The animals were observed immediately and after 4, 24, 48, and 72 h for evidence of abnormalities such as convulsions, prostrations, loss of body weight more than 2 g and death.

Intracutaneous (intradermal) reactivity test (ISO 10993-10)

This test was done to assess the potential of the material to produce irritation following intradermal injection of the material extract. The test samples were prepared by washing the samples in sterile normal saline for three wash cycles, with each cycle lasting for 10 min. The samples were dried at 37°C for 24 h. Two grams each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and cotton seed oil for 72 h at 50°C. Then, 0.2 mL (per each site) of the extract was injected into five sites with appropriate controls in randomly selected healthy, active New Zealand white rabbits. The animals were observed for erythema and edema which was scored as 0 (nil), 1 (very slight), 2 (well-defined edema/erythema), 3 (moderate), and 4 (severe). The requirement of the test is met if the difference between the test sample mean score and control sample mean score is 1.0 or less. All the procedures were done aseptically.

Implantation study

This study was done to evaluate calcification potential, immunogenic potential, and tissue response by subcutaneous implantation for 60 days in 3-week-old male Wistar rats. Healthy animals were randomly assigned into Glut BP and Dcl BP groups with each group consisting of five animals. The animals were subcutaneously implanted under the dorsal skin with evenly placed six implants of the same treatment group. The animals were observed daily for 60 days and at the end of which they were killed. The serum was collected for estimating antibody response against bovine pericardial proteins. One set of explants (n = 15) were used for calcium estimation and another set (n = 15) was processed for histopathological evaluation.

Antibody response

The antibody response (IgG, IgM, and IgA) elicited in different groups of animals implanted with Glut BP and Dcl BP samples was assessed using indirect ELISA. For this, extractable protein of fresh bovine pericardium was coated in the 96 well ELISA immuno plate (F96 MAXISORP, NUNC) in PBSN (2 μg protein per well). Serum samples at 1:1000 dilution prepared in blocking buffer from animals belonging to the above groups (n = 5) was used as primary antibody. Serum from animals which is immunized with extractable protein from fresh bovine pericardium was treated as positive control. Rabbit polyclonal to rat IgG + IgM + IgA conjugated with HRP (Abcam ab8521) was used as secondary antibody. After the

reaction, the ELISA plates were read with microplate reader Expert Plus (ASYS Hitech GmbH, Austria). OD readings of micro wells without secondary antibody were taken as the negative control. OD values subtracted from the mean negative control values were used for comparison.

Calcification response

Calcium estimations from explanted samples were done as per the procedure reported earlier.[7] In brief, the explanted Glut BP and Dcl BP samples (n = 15, three samples from each animal) were digested in 2 mL of 2 N HCl for 48 h at 60°C. The supernatant was neutralized with 2 N NaOH followed by the estimation of calcium using the colorimetric method of O-Cresolphthalein complexone obtained as a standard kit. OD readings were taken at 578 nm using a Shimadzu UV-1700 spectrophotometer, Japan.

Tissue response

The explanted Glut BP and Dcl BP samples (n = 15, three samples from each animal) were fixed in 10% buffered formalin, embedded in paraffin, and 5 μm sections were made. The sections were stained by hematoxylin and eosin and Von Kosa stain. A qualitative assessment was made for peri-implant necrosis, inflammatory response, peri-implant fibrosis, tissue incorporation, angiogenesis in the implanted material and calcification.

Statistics

All quantitative estimations are expressed in mean ± SD. Significance testing for differences of means between groups is carried out using the unpaired Student-t test for equal or unequal variance depending on the homogeneity of variance tested using the F-test.. Statistically significant difference between means was assumed whenever P-value was less than 0.05.

RESULTS

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Confirmation of decellularization

Nuclear remnants could not be demonstrated both by hematoxylin and eosin as well as by Hoechst 33258 staining [Figure 1], indicating satisfactory decellularization. DNA extraction yielded a residual DNA content of 7 ± 3.5 μg/mL in decellularized bovine pericardium compared to 97 ± 2 μg/mL in fresh bovine pericardium (triplicate samples). Extractable DNA from decellularized tissue could not be demonstrated in 1% agarose gel electrophoresis probably because of extensive DNA fragmentation [Figure 2]. Structurally, decellularized pericardium mainly consisted of well-separated collagen bundles with a few elastin fibers as demonstrated by Movats Pentachrome staining.

Collagenase susceptibility

Data on residual weight of different groups following Collagenase II digestion are given in Table 1 and data on degradation are given in Table 2.

Following Collagenase type II digestion for 24 h, Dcl BP samples had 90.2% degradation with a residual weight of 4.9 ± 0.4 mg. Glut BP group had maximum Collagenase resistance with minimum weight loss of around 1 mg (2%). At 72 h residual weight Dcl BP and Glut BP groups showed a residual weight of 1.8 ± 0.3 mg (96.5% degradation) and 49 ± 1 mg (2% degradation), respectively. At 7 days, the residual weights were 1.3 ± 0.25 mg and 48 ± 1 mg for Dcl BP and Glut BP groups, respectively. At this period, the rate of weight loss has slowed down in the Dcl BP group with only an additional 1% weight loss was noticed in

about 7 days. The Glut BP group continued to lose weight at very low rate reaching about 4% at 7 days.

In vitro response

Cytotoxicity: A direct contact Dcl BP samples were seen noncytotoxic to L929 fibroblasts by a direct contact method [Figure 3]. The Glut BP sample was seen moderately cytotoxic probably because of residual glutaraldehyde. The positive control was severely cytotoxic, and the negative control was noncytotoxic.

Cytotoxicity testing on extract The MTT assay of L929 cells after contact with 50% and 25% extracts of Dcl BP showed 96% and 97% metabolic activities, respectively, compared to Glut BP which had 14% and 15.1% metabolic activities, respectively. This clearly showed that the extract of Glut BP samples has significantly affected the metabolic activity of fibroblast cells and hence it is toxic in nature compared to Dcl BP.

Macrophage activation Cytokine release profile of activated macrophage in response to exposure to different groups is given in Table 2. The positive control group showed the highest cytokine response. With respect to the TNFα and IL1β, highest macrophage activation was seen in the Glut BP group. This was seen statistically significant compared to the Dcl BP group. In the case of IL6, the Dcl BP group showed a higher response when compared to the Glut BP group ($P = 0.004$).

In vivo response

Acute systemic toxicity testing (ISO 10993-11) Administration of cotton seed oil extract of the Glut BP sample through an intraperitoneal route did not produce a systemic toxic effect evidenced as loss of body weight more than 2 g, the presence of any abnormalities or death. However, intravenous administration of physiological saline extract of the Glut BP sample produced abnormalities and death immediately after injection in three mice, indicating systemic toxicity of this sample. The control animals did not show any signs of systemic toxicity. The cotton seed oil extract and physiological saline extract of the Dcl BP sample did not produce any abnormalities, loss of weight or death following intraperitoneal and intravenous administration, respectively. This indicated that the Dcl BP extract do not cause systemic toxicity.

Intracutaneous (intradermal) reactivity test (ISO 10993-10)

The physiological saline extract of the Glut BP sample produced an average irritation score of 1.18 which is a positive reactivity for this test. At the same time, the cotton seed oil extract showed 0 score of irritation indicating no intracutaneous irritation. The physiological saline and cotton seed oil extract of Dcl BP samples produced irritation scores of 0.3 and 0.1, respectively, which indicated that the samples are nonirritating as per the set criteria for this test.

Implantation study

Animal implantation was uneventful and all animals in the groups Glut BP and Dcl BP survived the duration of the study uneventfully.

Antibody response

Antibody response against extractable protein from fresh bovine pericardium elicited in animals implanted with different treatment groups is given in Table 2. The Dcl BP group had significantly lower antibody response against fresh bovine pericardial protein compared to the Glut BP group ($P < 0.05$). This indicated

that glutaraldehyde treatment which is done to reduce immune response of xenografts has failed to reduce it in comparison to decellularization.

Calcification response

The result of calcification experiment is given in Table 2. The Dcl BP group explant had significantly lower calcium content of 157 ± 31 μg/100 mg dry weight of tissue in comparison to 434 ± 84 μg/100 mg of dry weight of the Glut BP explant.

Tissue response

The tissue response studied on hematoxylin and eosin stained paraffin sections is presented in Figure 4. The sections were qualitatively assessed for peri-implant necrosis, inflammatory response, calcification, peri-implant fibrosis, tissue incorporation, and angiogenesis into the implanted material. The Dcl BP implant did not show any peri-implant necrosis. A thin fibrous capsule could be appreciated around the implant. Mild-to-moderate diffuse infiltration of mononuclear cells into the implant is noticed indicating chronic inflammation. Macrophages could be identified at the site of implant degradation. Implant calcification could not be observed. Implant showed excellent ingrowth of cells with fibroblast morphology with evidence of newly laid collagen. Angiogenesis is noticed in the implant as well at the periphery.

Glut BP explants showed a thick capsule around the implant. Certain areas showed peri-implant necrosis. Angiogenesis was seen only in the periphery of the implant. Focal mild-to-moderate chronic inflammation observed as mononuclear cell infiltration could be seen at the periphery of the implant. The implant interior was remarkably acellular. Calcium deposit could be observed in many sections.

DISCUSSION

Go to: Go to:

In this study, it is attempted to demonstrate that glutaraldehyde treatment of bovine pericardium induces toxic response which will ultimately affect its long-term performance in clinical situation. This is because of its cytotoxicity, persistent inflammation, immunogenicity, calcification, and lack of remodeling. It is reasoned that decellularization of bovine pericardium will bring about positive remodeling of the implant which is brought about by its susceptibility to matrix metalloproteinase which promotes intraimplant host tissue incorporation, new matrix deposition, and angiogenesis besides its low immunogenicity, inflammatory potential, and calcification potential. In this study, a commercially available clinically used glutaraldehyde-treated bovine pericardium was studied and hence it was not further characterized. Decellularized tissue produced by a nondetergent based decellularization method was used for comparison. The decellularization method employed was found adequate as nuclear remnants could not be detected by routine HE staining or by nuclear stain Hoechst 33258. Polyacrylamide gel electrophoresis of residual DNA could not demonstrate DNA bands indicating extensive DNA fragmentation.

The data clearly indicated that the glutaraldehyde-treated bovine pericardium is resistant to Collagenase which is a major enzyme in the body responsible for extracellular matrix remodeling. In the rat subcutaneous implantation model, this was seen as acellular areas in the implants indicating the absence of tissue penetration into the implant. In comparison decellularized bovine pericardium was seen susceptible to Collagenase enzyme and correspondingly the *in vivo* data showed excellent host tissue incorporation into the implant.

In vitro data such as direct cytotoxicity as well as toxicity of extract clearly suggested cytotoxic nature of

the glutaraldehyde-treated bovine pericardium. This is clearly correlated in rat implantation studies where it was observed as areas of peri-implant necrosis around Glut BP samples. Whereas Dcl BP samples or its extracts were seen to be noncytotoxic and subsequently peri-implant necrosis was absent in the rat study. The intracutaneous irritation study and systemic toxicity also showed that the physiological saline extract of the glutaraldehyde-treated bovine pericardium is of toxic nature. Interestingly, only the water-soluble extract was observed as toxic whereas as the cotton seed oil extract was seen nontoxic. Earlier studies have reported on the cytotoxic nature of glutaraldehyde-treated tissue without indicating which fraction is cytotoxic.[8] Macrophages as inflammatory cells as well as antigen-presenting cells have an important role in *in vivo* response against biomaterials. Hence a macrophage activation study was conducted. This study showed a definite increase in the release of inflammatory cytokines such as TNFα and IL1β in Glut BP samples compared to Dcl BP samples. This clearly indicated that the glutaraldehyde-treated bovine pericardium activates macrophages significantly in comparison to decellularized bovine pericardium. Increased inflammatory response noticed in Glut BP samples of a rat implantation study might be a reflection of what is observed in *in vitro*. Interestingly, IL6 release was seen higher in the Dcl BP sample compared to Glut BP samples. In contrast to the above observations, a study showed no significant variation between *in vitro* cytokine release of inflammatory and anti-inflammatory cytokines in response to different polymeric materials.[9]

In vivo data such as antibody response, calcification response, and tissue response demonstrated better biocompatibility for decellularized bovine pericardium compared to glutaraldehyde-treated one. For studying the immune response, focus was given on antibody response elicited against extractable protein from fresh pericardium as it was assumed that in processed bovine pericardium the residual protein is the major immunogen. Since there was complete fragmentation of DNA following decellularization, immunogenicity on this account was not anticipated. DNA fragments less than 300 bp is reported to be nonimmunogenic.[10] Decellularized bovine pericardium had significantly reduced antibody response compared to glutaraldehyde-treated one. This indicated that glutaraldehyde treatment is not necessarily immunosuppressive. A study on antigenicity of glutaraldehyde-treated bovine serum albumin (BSA) reported that glutaraldehyde caused intermolecular cross-linkages forming soluble aggregates; such modified proteins were seen antigenic and produced antibodies against antigenic determinant of BSA as well as against newly acquired groups arising from this modification.[11] The increased antibody response seen in the glutaraldehyde-treated samples might be due to formation of soluble aggregates of BSA. *In vivo* calcification was seen directly linked to the extent of glutaraldehyde treatment. Glutaraldehyde-treated samples were seen most calcifying as previously reported.[12] Decellularized pericardium had minimum calcification. A previous study has reported direct relation between calcification and antibody response.[13] This relationship was noticed here also. Decellularized pericardium and glutaraldehyde-treated pericardium presented two extreme tissue responses. Glutaraldehyde-treated pericardium showed no host cell incorporation into implant and a thicker fibrous tissue capsule separated the implant from the host tissue. The presence of mild-to-moderate chronic inflammation is noticed in the implant periphery. There is calcification as well as significant antibody response in this group. These conditions may not favor a positive tissue remodeling.[10] Similar observation was previously reported for glutaraldehyde-treated tissue.[8] Decellularized bovine pericardium showed contrasting and desirable tissue response such as thin fibrous capsule, undetectable calcification, implant degradation with laying of new collagen, and mild chronic inflammation. All the above observations point to better implant remodeling.[14] It is assumed that the presence of mononuclear cells in this group should not be of concern since the antibody response elicited in this group was significantly lower compared to the Glut BP group. In contrast the observations noted in the glutaraldehyde-treated group such as thicker fibrous capsule, implant

calcification, moderately intense mononuclear cell infiltration in the periphery and angiogenesis restricted to implant periphery and visible lack of host cell incorporation into implant are not conducive for the sustained implant function. Hence the observations made in glutaraldehyde-treated bovine pericardium are not congenial for an acceptable tissue compatibility as well as implant remodeling which is essential for successful long-term clinical performance of a surgical implant.

CONCLUSION

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Thus, it is concluded that glutaraldehyde-treated bovine pericardium incites significant toxic, inflammatory, immune, and calcification responses which is not desirable for a long-term function as an implant. Furthermore, glutaraldehyde also prevents tissue remodeling on account of its resistance to matrix metalloproteinase resulting in the implant remaining as a separate entity in the body without physiologically integrating into the host.

ACKNOWLEDGMENTS

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This work was supported by a grant from Department of Biotechnology, Government of India. The authors gratefully acknowledge the support of the Director, the Head, and other colleagues in Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, India.

Footnotes

Go to: Go to:

Source of Support: Department of Biotechnology, Government of India

Conflict of Interest: No conflict of interest.

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Go to: Go to:

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Figures and Tables

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Figure 1

Open in a separate window

Confirmation of decellularization with Hoechst 3328 staining. (a) Decellularized pericardium with no nuclear remnants. (b) Normal pericardium showing intact nucleus (Hoechst 33258, 400×)

Figure 2

Open in a separate window

Extractable DNA from decellularized bovine pericardium; first lane: 100 bp DNA ladder, second and third lanes: DNA from decellularized bovine pericardium, fourth lane: DNA from fresh bovine pericardium

Table 1

Residual weights in different groups with time following Collagenase type II digestion

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Table 2

Toxic response (*in vitro* and *in vivo*) of Glut BP compared to Dcl BP

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Figure 3

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(a) Glut BP sample showing moderate cytotoxicity (b) Del BP sample showing noncytotoxic nature

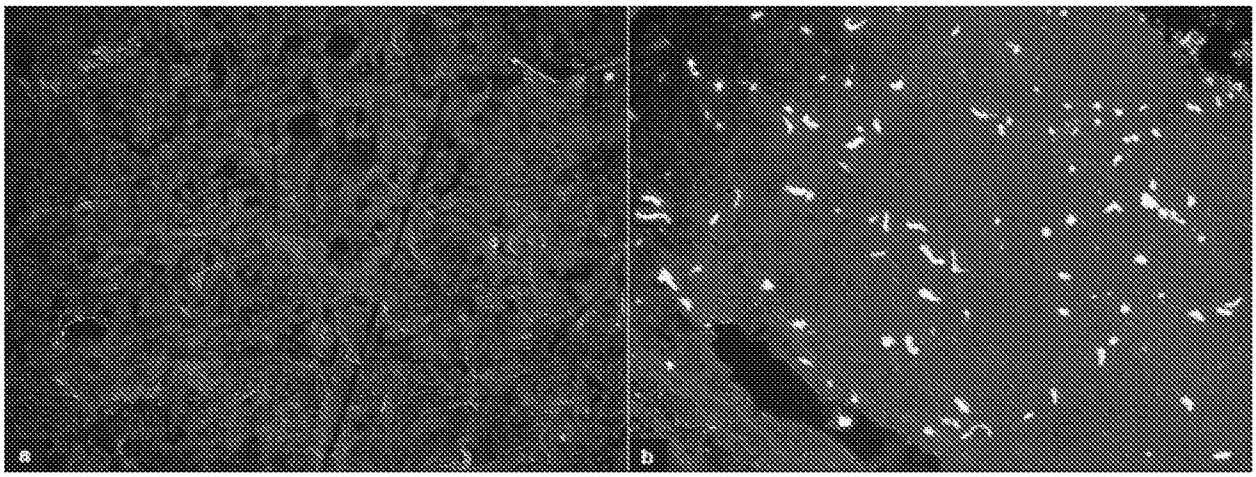
Figure 4

Open in a separate window

Tissue response to different implants at 60 days in rat subcutaneous implantation. (a) Del BP group minimum inflammatory response and uniform host cell incorporation and angiogenesis (arrow) within implant H and E, ×400. (b) Glut BP group, inflammatory response (arrow) in the interphase and remarkably acellular interior (H and E, ×200)

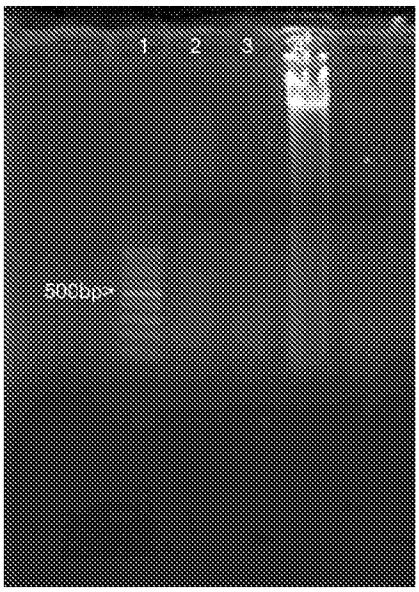
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Figure 1



Confirmation of decellularization with Hoechst 3328 staining. (a) Decellularized pericardium with no nuclear remnants. (b) Normal pericardium showing intact nucleus (Hoechst 33258, 400×)

Figure 2



Extractable DNA from decellularized bovine pericardium; first lane: 100 bp DNA ladder, second and third lanes: DNA from decellularized bovine pericardium, fourth lane: DNA from fresh bovine pericardium

Table 1

Residual weights in different groups with time following Collagenase type II digestion

Residual weight (mg)	24 h	72 h	7 days
Dcl BP	4.9 ± 0.4*	1.8 0.3*	1.3 ± 0.2
Glut BP	49 ± 1*	49 1	48 ± 1

* Statistically significant at P < 0.05. Comparison was made between groups and between different time periods of the same group

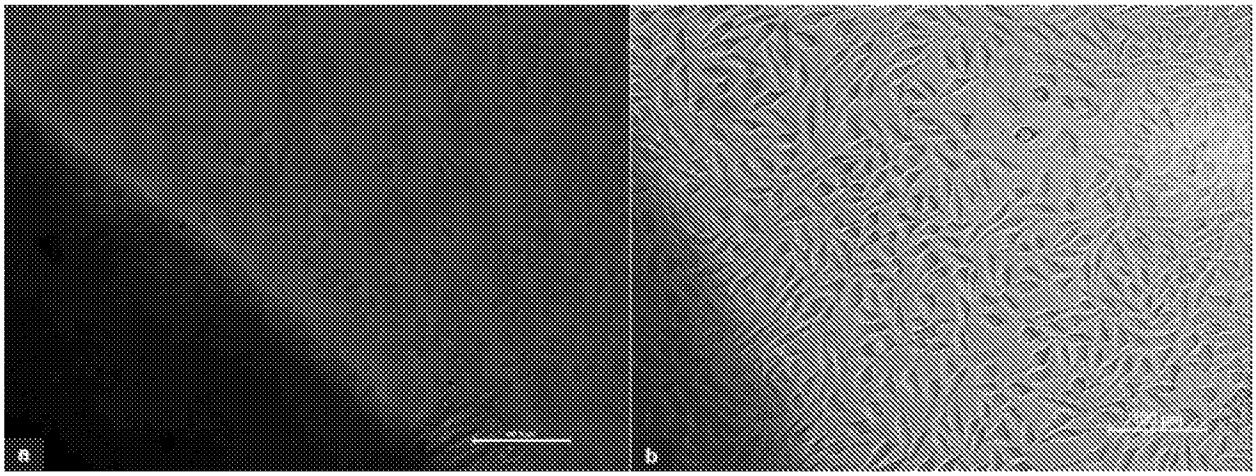
Table 2

Toxic response (*in vitro* and *in vivo*) of Glut BP compared to Dcl BP

Treatment groups	Dcl BP	Glut BP
Susceptibility to Collagenase type 2 (*in vitro*) %	90.2% degradation in 24 h; 96.5% degradation in 72 h; 97.4% in 7 days	2% degradation in 24 h, 2% in 72 h; 4% in 7 days
In vitro cytotoxicity to L929 fibroblast cells	Noncytotoxic	Moderately cytotoxic
In vitro cytotoxicity of extract	Noncytotoxic	Cytotoxic
In vitro Macrophage activation**		
IL1 beta (pg)	82.4 ± 4.1*	144 ± 7.4*
IL6 (pg)	59.1 ± 3.6*	46 ± 0.72*
TNF alpha (pg)	45.3 ± 6.6*	267 ± 22.9*
Systemic toxicity		
Intravenous route	Nontoxic	Toxic
Intraperitoneal route	Nontoxic	Nontoxic
Intracutaneous irritation		
Cotton seed oil extract	Nontoxic	Nontoxic
Physiological saline extract	Nontoxic	Toxic
In vivo antibody response to BP proteins (OD)	0.83 ± 0.08*	1.11 ± 0.16*
In vivo calcification response (μg/100 mg dry weight)	157 ± 31*	434 ± 84*
Tissue response		
Necrosis	Absent	Present
Inflammation	Mild to moderate, diffuse	Focal, mild to Moderate Thick
Fibrosis (peri-implant capsule)	Thin	
Tissue incorporation	Present	Absent
Angiogenesis within implant	Present	Focal deposit
Calcification	Not detectable	seen

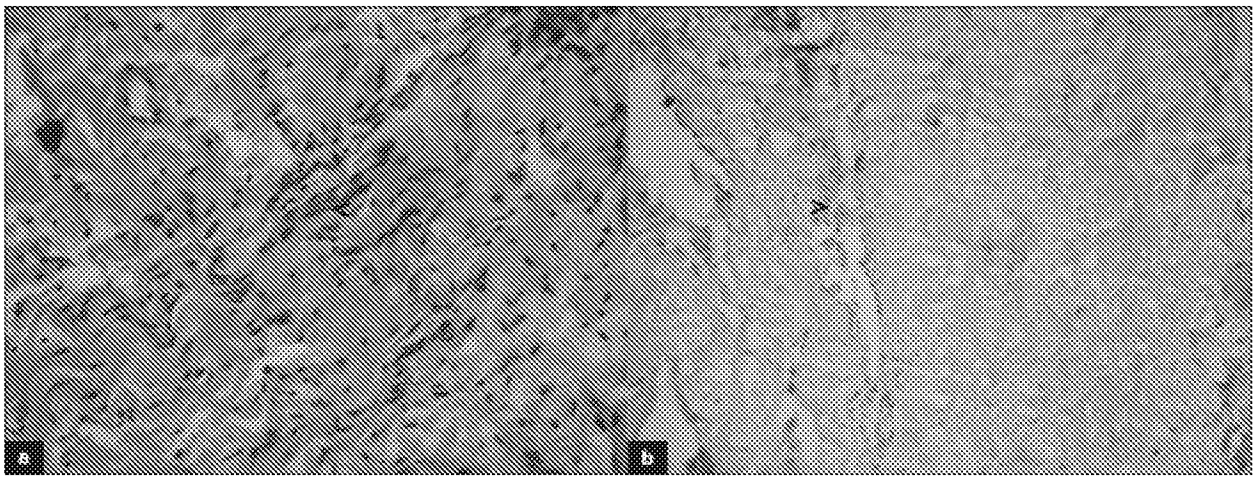
*Statistically significant difference at $P < 0.05$. **LPS activation caused cytokine release at, IL1β: 255 pg; IL6: 280 pg, and TNFα: 346 pg.

Figure 3



(a) Glut BP sample showing moderate cytotoxicity (b) Dcl BP sample showing noncytotoxic nature

Figure 4



Tissue response to different implants at 60 days in rat subcutaneous implantation. (a) Dcl BP group minimum inflammatory response and uniform host cell incorporation and angiogenesis (arrow) within implant H and E, ×400. (b) Glut BP group, inflammatory response (arrow) in the interphase and remarkably acellular interior (H and E, ×200)

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	1	of	1	GROUP ART UNIT: 1651	EXAMINER: R. A. Davis

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		6,485,969	B1	Asem et al.	11-26-2002

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		CN	1791331	A	Anthrogenesis Corp	06-21-2006	Y – Machine Translation
		CN	102227225	A	Univ California	10-26-2011	Y – Machine Translation

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		DUAN et al., Preliminary study on porous scaffold prepared with decellularized artery. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2010 Sep;24(9):1052-7.	Y – Abstract Only
		MENG et al., An experimental study of extracellular matrix of conjunctiva. Journal of Injusries and Occupational Diseases of the Eye with Ophthalmic Surgeries. 2007 May;1:26-8.	Y – Abstract Only
		UMASHANKAR et al., Glutaraldehyde Treatment Elicits Toxic Response Compared to Decellularization in Bovine Pericardium. Toxicol Int 2012;19(1):51-8.	

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EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

DOCKET NO.: S1860.70000US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	R. A. Davis
Art Unit:	1651

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STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed before the mailing of a first Office Action after the filing of a Request for Continued Examination under 37 C.F.R. § 1.114.

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

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An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Edward R. Gates/
Edward R. Gates, Reg. No. 31,616
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Telephone: (617) 646-8000

Docket No.: S1860.70000US01
Date: Oct 22, 2018

Electronic Patent Application Fee Transmittal

Application Number:	14759895
Filing Date:	08-Jul-2015
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Filer:	Edward R. Gates/Eileen MacKenzie
Attorney Docket Number:	S1860.70000US01

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	2253	1	700	700
Miscellaneous:				
RCE- 1ST REQUEST	2801	1	650	650
		Total in USD ($)		**1350**

Electronic Acknowledgement Receipt

EFS ID:	34077876
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Eileen MacKenzie
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	22-OCT-2018
Filing Date:	08-JUL-2015
Time Stamp:	18:58:52
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

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Payment Type	CARD
Payment was successfully received in RAM	$1350
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	Amendment Submitted/Entered with Filing of CPA/RCE	1	1

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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Publication number: CN1791331 A
Publication country: CHINA
Publication type: UNEXAMINED APPLIC. OPEN TO PUBLIC INSPECTION
Publication date: 20060621
Application number: CN20038012096
Application date: 20030326
Priority: US20020106653 20020326 US20020106653;
Assignee[std]: ANTHROGENESIS CORP ;
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Inventor[std]: HARIRI ROBERT J KAPLUNOVSKY AL ;
International class[8]: A01N27/00; A61F2/00; A61K35/54; A61L27/36; C12N5/00; C12N5/08;
Cooperative Patent class: C12N5/0068 ; A61K35/50 ; A61L27/3604 ; A61L27/3683 ; A61L27/3691 ; A61L2430/40 ; C12N2533/92 ; Y10T442/2525 ;
Family members: AU2003226018 AA AU2003226018 BB AU2008202604 AA AU2008202604 BB AU2011201662 AA AU2011201662 BB CA2479903 AA CA2479903 C CN1791331 A DE60346784 D1 EP1575572 A2 EP1575572 A3 EP1575572 A4 EP1575572 B1 EP2700309 A1 EP2702871 A1 ES2526088 T3 HK1085384 A1 HK1085384 A1 HK1085384 A3 HK1085384 B HK1192693 A1 IL164220 A0 IL164220 A1 JP2006507851 T2 JP2010265298 A2 JP2014138590 A2 JP4615223 B2 JP5931327 B2 JP5993387 B2 KR100999247 B1 KR20050002906 A MXPA04009188 A1 NZ535799 A US2003187515 AA US2004048796 AA WO03082201 A2 WO03082201 A3 ZA200407650 A ZA200407650 A
Title: COLLAGEN BIOFABRIC AND METHODS OF PREPARATION AND USE THEREFOR
Title: 胶原生物织物以及其制备和使用方法

Abstract:

本发明涉及从羊膜制得的胶原膜，此处称为胶原生物织物。本发明的胶原生物织物具有天然的未处理的羊膜的结构完整性，即天然的三级和四级结构。本发明提供了通过对羊膜进行去细胞化而从胎盘膜制备胶原生物织物的方法，优选地从具有绒毛膜和羊膜的人胎盘膜制备。在一个优选的实施方案中，羊膜完全地进行去细胞化。本发明的胶原生物织物在内科和外科领域有着许多的应用，包括例如血管修复、血管的构建和替换、腱和韧带的替换、创伤敷裹、外科移植物、眼科用途、缝合等等。该生物织物的优点部分地是由于其物理特性，例如生物机械强度、屈曲性、可缝合性和低免疫原性，特别是当其来源自人胎盘时。

The present invention relates to the amniotic membrane prepared from collagen, the collagen referred to herein as biological fabric. Structural integrity of the collagen biological fabric of the present invention has a natural untreated amniotic membrane, the natural tertiary and quaternary structure. The present invention provides a decellularized amniotic were prepared from the biological fabrics placenta collagen membrane, preferably having from human chorionic placenta and amniotic membranes. In a preferred embodiment, the amniotic membrane were completely decellularized. Collagen biological fabric of the present invention has many areas of

medical and surgical applications, including for example, vascular repair, construction and replacement, tendon and ligament replacement blood vessels, wound dressings, surgical implants, ophthalmic use, stitching and the like. The advantages of the biological fabric was due to the physical properties, such as bio-mechanical strength, buckling resistance, and low-seamable immunogenicity, especially when the source is from human placenta.

Claims:

1.从具有羊膜和绒毛膜的胎盘制备胶原生物织物的方法，包括：(a)分离羊膜和绒毛膜；和(b)将羊膜进行去细胞化，以致羊膜不与薛接触。

5 2.权利要求1的方法，其进一步包括洗涤和干燥去细胞化的羊 膜。

3.权利要求1的方法，其中胎盘为人胎盘。

4.权利要求1的方法，其中胎盘来自经历了剖腹产术分娩或者自 然分娩的女性人类。

10 5.权利要求1的方法，其中该方法另外包括确定胎盘膜来自已经 检测了至少一种传染病的供体的步骤。

6.权利要求1的方法，其中供体为女性人类。

7.权利要求1的方法，其中胎盘在生育的48小时之内提供。

8.权利要求1的方法，其中该方法另外包括在步骤(a)之后和步 15 骤（b)之前将羊膜贮存直至72小时的步骤。

9.权利要求8的方法，其中所述贮存包括将在步骤（a)中获得的 羊膜冷藏直至5天。

10.权利要求1的方法，其中步骤（b)中的羊膜的去细胞化包括从 羊膜上去除所有可见的细胞材料和细胞碎片。

20 11.权利要求1的方法，其中步骤（b)中的羊膜的去细胞化包括从 羊膜的母体侧和羊膜的胎儿侧去除所有可见的细胞材料和细胞碎片。

12.权利要求1的方法，其中步骤（b)中的羊膜的去细胞化包括物 理性刮削所述的膜。

13.权利要求1的方法，其中步骤（b)中的羊膜的去细胞化包括用 25 含有去污剂的溶液将羊膜进行去

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1 collagen prepared from biological fabric having a placenta amnion and chorion, comprising: (a) separating amnion and chorion; and (b) the decellularized amniotic membrane, amniotic membrane that does not Xue contacts.

5 2. The method of claim 1, further comprising washing and drying the decellularized amniotic.

3. The method of claim 1, wherein the human placenta Placenta.

1 of 4 claim wherein the placenta from cesarean delivery experienced a natural childbirth or female human.

10 5. The method of claim 1, wherein the method further comprises determining the placenta membrane has been detected from the body of at least one step for infectious diseases.

6. The method of claim 1, wherein the donor is a female human.

7. The method of claim 1, wherein the placenta provided within 48 hours of birth.

1 8. The method of claim wherein the method further comprises, after steps (a) and step 15 step (b) prior to the amniotic stored for up to 72-hour procedures.

9. The method of claim 8, wherein the reservoir comprises a refrigerated amniotic membrane obtained in step (a), up to 5 days.

10. The method of claim 1, wherein step (b) in the decellularized amniotic including the removal of all visible cellular material and cellular debris from the amniotic membrane.

细胞化。

14.权利要求13的方法，其中含有去污剂的溶液为含有0.01-1. 0%—水合脱氧胆酸钠的溶液。

15.权利要求13的方法，其中在含有去污剂的溶液中的去污剂选 自非离子去污剂、Triton X-100、阴离子去污剂和十二烷基硫酸钠或 30 者其组合。

16.权利要求12的方法，其中所述的物理性刮削包括用细胞刮刀 进行刮削。

17.权利要求1的方法，其中步骤(b)中的羊膜的去细胞化在无菌 溶液中进行。

18.权利要求2的方法，其中羊膜的洗涤在无菌溶液中进行。

19.权利要求2的方法，其中去细胞化的羊膜的干燥于大约351C 5 -大约501C的温度进行。

20.从具有羊膜和绒毛膜的胎盘制备羊膜层压制品的方法，其包 括：(a)分离羊膜和绒毛膜；(b)将羊膜进行去细胞化；和 10 (C)将至少两层去细胞化的羊膜以相互接触的方式层叠在一起， 从而形成羊膜层压制品。

21.权利要求20的方法，其进一步包括在步骤（b)之后和步骤（c) 之前洗涤去细胞化的羊膜至少一次。

22.权利要求20的方法，其进一步包括干燥去细胞化的羊膜层压 15 制品。

23.权利要求20的方法，其进一步包括将至少两层所述的羊膜层 压制品装配成复杂的三维支架。

24.含有脱水的、去细胞化的且无基质的羊膜的胶原生物织物， 其中所述的羊膜具有天然的三级和四级结构。

20 25.去细胞化的且无基质的胶原生物织物，其含有胶原、弹性蛋 白和纤连蛋白。

26.用权利要求1的方法制备得到的胶原生物织物。

27.权利要求24的胶原生物织物，其中羊膜为人羊膜。

28.权利要求24-26中任何一项的胶原生物织物，其中生物织物 25 的厚度为大约10-40微米。

29.权利要求24-26中任何一项的胶原生物织物，其中生物织物 进一步用一种或多种生物分子进行浸渍。

Method 1

20 11. claim, wherein step (b) in the decellularized amniotic including the removal of all visible cellular material and cells from the fetal side of the amnion and amniotic maternal side of debris.

12. The method of claim 1, wherein step (b) in the decellularized amniotic membrane comprises physical properties of the scrape.

13. The method of claim 1, wherein step (b) in the decellularized amniotic membrane comprises 25 containing a detergent solution decellularized amniotic membrane.

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14 The method of claim 13, wherein the solution containing the detergent containing 0.01-1 0 percent - Hydrated sodium deoxycholate solution.

15. The method of claim 13, wherein the detergent in the detergent-containing solution selected from non-ionic detergent, Triton X-100, an anionic detergent and ten 30, sodium lauryl sulfate, or by a combination thereof.

16. The method of claim 12, wherein said physical scraping with a cell scraper includes a scraping.

17. The method of claim 1, wherein step (b) in the decellularized amniotic carried out in a sterile solution.

18. The method of claim 2, wherein the amniotic membrane was washed in a sterile solution.

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19 The method of claim 2, wherein the decellularized amniotic membrane is dried at about 351C 5 - for about 501C temperature.

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20 prepared from the amniotic membrane laminate having a placenta amnion and chorionic method, comprising: (a) separating amnion and chorion; (b) the decellularized amniotic membrane; and 10 (C) at least two layers of decellularized amniotic membrane in contact with each other stacked together to form the amniotic membrane laminate.

20. The method of 21 claim, further comprising the step (b) and after step (c) washed before

30.权利要求29的胶原生物织物，其中生物分子选自抗生素、激 素、生长因子、抗肿瘤剂、抗真菌剂、抗病毒剂、疼痛药物、抗组胺 30 剂、抗炎剂、抗感染剂、伤口愈合剂、伤口封闭剂、细胞吸引剂和支 架试剂。

31.权利要求29的胶原生物织物，其中生物织物进一步用一种或 多种小分子进行浸渍。

32.权利要求24-26中任何一项的胶原生物织物，其中进一步在 生物织物上移植细胞，从而所述细胞是均一的和汇合的。

33.权利要求32的胶原生物织物，其中细胞为人干细胞或人分化 5 的成熟细胞。

34.权利要求24-26中任何一项的胶原生物织物，其进一步含有 一种或多种治疗剂。

35.权利要求34的胶原生物织物，其中所述的治疗剂选自激素、 多肽、抗生素、抗真菌剂和酶。

10 36.权利要求24-26中任何一项的胶原生物织物，其进一步含有 一种或多种水凝胶组合物。

37.权利要求36的胶原生物织物，其中水凝胶组合物含有选自聚 乙烯醇、聚乙二醇、透明质酸、葡聚糖和其衍生物或类似物的聚合物。

38. 含有权利要求24-26中任何一项的胶原生物织物的三维支 15 架。

39.权利要求38的三维支架，其中支架为管。

40.权利要求38的三维支架，其进一步含有一种或多种水凝胶组 合物。

41.权利要求40的三维支架，其中水凝胶组合物含有选自聚乙烯 20 醇、聚乙二醇、透明质酸、葡聚糖和其衍生物或类似物的聚合物。

42.用权利要求20的方法产生出的羊膜层压制品。

43.含有至少两层权利要求24-26中任何一项的胶原生物织物的 羊膜层压制品。

44.含有权利要求24-26中任何一项的胶原生物织物的羊膜层压 25 制品。

45.权利要求42-44中任何一项的羊膜层压制品，其进一步含有 一种或多种水凝胶组合物。

46.权利要求45的羊膜层压制品，其中水凝胶组合物含有选自聚 乙烯醇、聚乙二醇、透明质酸、葡聚

decellularized amniotic membrane at least once.

22. The method of claim 20, further comprising drying the decellularized amniotic membrane laminate 15 products.

20 23 The method of claim further comprising at least amniotic assembled layers laminated article according to complex three-dimensional scaffold.

24. Containing dehydrated, decellularized matrix and no biological amnion collagen fabric, wherein said amniotic membrane has a natural tertiary and quaternary structure.

20 25. decellularized matrix of collagen and no biological fabric containing collagen, elastin and fibronectin protein.

26. With claim 1, obtained by preparing collagen biological fabric.

27. Biological collagen fabric of claim 24, wherein the human amniotic amnion.

28. 24-26 biological collagen fabric according to any one of the preceding claims, wherein the thickness of the fabric 25 is about the biological 10-40 microns.

29. 24-26 collagen claimed in any one of claims Biological fabric, wherein the fabric further biological biomolecules with one or more impregnation.

30. Biological collagen fabric of claim 29, wherein the biomolecule is selected from antibiotics, hormones, growth factors, anti-tumor agents, antifungal agents, antiviral agents, pain medications, anti-histamines 30, anti-inflammatory agents, anti-infective agents, wound healing agents, wound sealant, cell attractant and bracket reagents.

31. Biological collagen fabric as claimed in claim 29, wherein the further biological fabric with one or more small molecule impregnation.

32. 24-26 biological collagen fabric according to any one of claims, wherein the further biologically transplanted cells in the fabric, so that the cells are uniform and confluent.

33. Collagen biological fabric of claim 32, wherein the cells are human stem cells or human cell

糖和其衍生物或类似物的聚合物。

30 47.含有权利要求24-26中任何一项的胶原生物织物的外科移植 物。

48.在外科程序中使用权利要求47的外科移植物的方法，其中所 述的外科移植物直接应用于受试者的外科部位。

49.权利要求48的方法，其中所述的外科部位选自眼、皮肤、腹 部的浆膜面、胸腔的浆膜面、浆膜心包、口腔的粘膜面、鼻腔的粘膜 面、呼吸道的表面、胃肠道的表面、泌尿生殖道的表面。

5 50.权利要求48的方法，其中所述的外科移植物应用于受试者身 体的内部部位。

51.权利要求48的方法，其中所述的外科移植物应用于受试者身 体的外部部位。

52.权利要求48的方法，其中所述的受试者为人。

10 53.在受试者中治疗和/或预防眼病的方法，其包括将权利要求 24-26中任何一项的胶原生物织物置于受试者的患病的眼表面上。

54.权利要求53的方法，其中眼病选自溃疡/穿孔、大泡性角膜 病变、眼部皮样囊肿/肿瘤、原发性翼状胬肉、持续性角膜上皮缺损、 急性和慢性碱灼伤、热灼伤、无虹膜、特应性角膜炎、特发性边缘干 15 细胞缺乏、角膜翳、新血管生成、类风湿性角膜融化、眼瘢痕性类天 疱疮、渗漏性滤过性水泡、暴露的Ahmed瓣膜管、具有随后的睑球粘 连的沙雷氏菌蜂窝组织炎、急性和慢性Stevenson Johnson综合征。

55.在外科程序中使用权利要求24-26中任何一项的胶原生物织 物的方法，其中外科程序选自眼外科、心血管外科、牙周外科、神经 20 外科、牙外科和矫形外科。

56.在受试者中矫正尿失禁之中使用权利要求24-26中任何一项 的胶原生物织物的方法。

57.将治疗剂递送给受试者的方法，其包括使权利要求24-26中 任何一项的胶原生物织物与受试者接触。

25 58.权利要求56或57的方法，其中受试者为人。

59.权利要求57的方法，其中治疗剂选自抗生素、抗癌剂、抗菌 剂、抗病毒剂、疫苗、麻醉剂、镇痛药、抗哮喘剂、抗炎剂、抗抑郁 剂、抗糖尿病剂、

differentiation and maturation 5.

34. 24-26 biological collagen fabric according to any one of the preceding claims, further comprising one or more therapeutic agents.

35. Biological collagen fabric as claimed in claim 34, wherein said therapeutic agent is selected from hormones, polypeptides, antibiotics, antifungal agents, and enzymes.

10 36. 24-26 claimed in any one of the collagen fabric biological claims, further comprising one or more hydrogel composition.

36 37 Collagen biological fabric of claim wherein the hydrogel composition is selected from the group comprising polyvinyl alcohol, polyethylene glycol, hyaluronic acid, dextran or derivatives thereof, and like polymers.

38. 24-26 containing three-dimensional collagen support any one of the biological fabric of claim 15.

39. Scaffolds as claimed in claim 38, wherein a tube holder.

40. Scaffolds as claimed in claim 38, further comprising one or more hydrogel composition.

41. Scaffolds as claimed in claim 40, wherein the hydrogel composition comprises 20 selected from polyethylene glycol, polyethylene glycol, hyaluronic acid, dextran or derivatives thereof, and like polymers.

42. The method of claim 20, produced by the amniotic membrane laminate.

43. 24-26 comprising at least two claimed in any one of amnion collagen fabric laminate biological requirements.

44. 24-26 contain any one of amnion collagen biological fabric laminate article of claim 25.

45. 42-44 amniotic membrane laminate as claimed in any one of claims, further comprising one or more hydrogel composition.

46. 45 amniotic laminate of claim wherein the hydrogel composition is selected from the group comprising polyvinyl alcohol, polyethylene glycol,

抗精神病药、中枢神经系统刺激剂、激素、免疫抑制剂、肌肉松弛剂、前列腺素。

30 60.使用权利要求24-26中任何一项的胶原生物织物的方法，其 中胶原生物织物的制备时间包括胶原生物织物的水化。

61.权利要求60的方法，其中生物织物的水化包括用无菌盐溶液 进行水化。

62.权利要求60的方法，其中生物织物在使用之前水化至少2分 钟。

63.使用羊膜层压制品的方法，其进一步包括在层压制品上移植 5 活细胞。

64.权利要求63的方法，其中所述的活细胞选自成熟组织细胞和 干细胞。

65.权利要求64的方法，其中所述的干细胞为全能性的。

66.权利要求64的方法，其中所述的干细胞为多能性的。

10 67.权利要求64的方法，其中所述的干细胞为组织特异性的。

68.在受试者中处理或预防皮肤状况的方法，其包括使权利要求 24-26中任何一项的胶原生物织物与所述的皮肤状况接触。

69.权利要求68的方法，其中所述的皮肤状况选自皮肤损伤、皱 纹、细纹、皮肤变薄、降低的皮肤弹性、粗糙的皮肤、痤疮瘢痕、眉 15 间沟、切除性瘢痕、软组织缺损、先天的皮肤状况、变性的皮肤状况、 VII型胶原缺乏和阳光损害的皮肤。

70.权利要求68的方法，其进一步包括向受试者施用一种或多种 治疗剂以处理皮肤状况。

71.权利要求70的方法，其中所述的一种或多种治疗剂选自维生 20 素、矿物质、基于儿茶素的制剂和葡糖胺。

72.在受试者中治疗伤口的方法，其包括使权利要求24-26中任 何一项的胶原生物织物与所述的伤口接触。

73.权利要求72的方法，其中所述的伤口选自表皮伤口、皮肤伤 口、压力性溃疡、慢性伤口、急性伤口、外部伤口、内部伤口、先天 25 性伤口、烧伤伤口、外科伤口和伤口感染。

hyaluronic acid, dextran, and derivatives thereof or the like polymers.

30 47. contain any one of claims 24-26 biological collagen surgical graft fabric.

48. Use Rights surgical graft method of claim 47 in surgical procedures, a surgical graft wherein said applied directly to the surgical site subject.

49. The method of claim 48, wherein said surgical site is selected from eyes, skin, serosa of the abdomen, the chest serosa, serous pericardium, oral mucosal surface, nasal mucosal surface of the respiratory surface, surface, surface of the urogenital tract gastrointestinal tract.

5 50. The method of claim 48, wherein said surgical graft applied to the internal parts of the body of the subject.

51. The method of claim 48, wherein said surgical graft is applied to the external parts of the body of the subject.

52. The method of claim 48, wherein the subject is a human.

10 53. treating and / or preventing eye disease in a subject, which comprises 24-26 biological collagen fabric placed in any one of claims subject suffering on the ocular surface disease.

54. The method of claim 53, wherein the eye disease is selected from ulceration / perforation, bullous keratopathy, ocular dermoid cysts / tumors, primary pterygium, persistent corneal epithelial defect, acute and chronic alkali burns, thermal burns, aniridia, atopic keratitis, idiopathic edge 15 Stem cell deficiency, pannus, neovascularization, rheumatoid corneal melting, eye scarring pemphigoid, leaky filtration blisters, Ahmed valve tube is exposed, with subsequent symblepharon marcescens cellulitis, acute and chronic Stevenson Johnson syndrome.

55. Use in surgical procedures in a method as claimed in any one of 24-26 collagen bio-fabric, wherein the surgical procedure is selected from eye surgery, cardiovascular surgery, periodontal surgery, nerve 20 surgery, dental surgery and orthopedic surgery.

56. Correction of urinary incontinence among 24-26 use rights to any one of the biological collagen

74.在受试者中治疗烧伤的方法，其包括使权利要求24-26中任 何一项的胶原生物织物与所述的烧伤接触。

75.权利要求74的方法，其中所述的烧伤选自一度烧伤、二度烧 伤、三度烧伤、感染的烧伤伤口和烧伤伤口脓疱病。

30 76. 权利要求68、72或74的方法，其中受试者为人。

fabric requirements in a subject.

57. The delivery of therapeutic agents to the subject method, which claims 24 to 26 comprising biological collagen in contact with the fabric according to any one of the subject.

25 58. The method of claim 56 or 57, wherein the subject is a human.

59. The method of claim 57, wherein the therapeutic agent is selected from antibiotics, anti-cancer agents, antimicrobial agents, antiviral agents, vaccines, anesthetics, analgesics, anti-asthmatic agents, antiinflammatory, antidepressants, antidiabetic agents, antipsychotics, central nervous system stimulants, hormones, immunosuppressive agents, muscle relaxants, prostaglandins.

30 60. Use of the method according to any one of claim 24-26 collagen bio-fabric requirements, the time of preparation of collagen biological fabrics include fabric biological collagen hydration.

61. 60 The method of claim, wherein the biological hydration fabric comprising hydrated with sterile saline solution.

62. 60 The method of claim, wherein the biological fabric hydrated before use at least two minutes.

63. Amniotic laminate using method 5, further comprising living cells in the transplant laminate.

63 64 method of claim wherein the living cells are selected from adult tissue and stem cells.

65. The method of claim 64, wherein said stem cells are totipotent.

66. The method of claim 64, wherein said stem cells are pluripotent.

Method 64

10 67. claim, wherein said stem cells are tissue specific.

68. The method of treatment or prevention of a skin condition in a subject, which claims 24-26 comprising biological collagen fabric according to any one of the contact with the skin condition.

68 69 The method of claim wherein said skin condition is selected from skin damage, wrinkles,

fine lines, skin thinning, decrease of skin elasticity, rough skin, acne scars, Mei 15 ditch, scar removal, soft tissue defects, congenital skin condition, skin condition degeneration, VII collagen deficiency and sun damaged skin.

70. The method of claim 68, further comprising administering to the subject one or more therapeutic agents to treat skin condition.

70 71 method of claim wherein the one or more therapeutic agents selected from vitamin 20 vitamins, minerals, tea-based child pigment formulations and glucosamine.

method of treating a wound in a subject 72, which comprises contacting the wound with the claim in any one of the biological collagen fabrics 24-26.

73. The method as claimed in claim 72, wherein the wound is selected from skin wounds, skin wounds, stress ulcer, chronic wounds, acute wounds, external wounds, internal wounds, congenital 25 wound, burn wounds, surgical wounds and wound infections.

method of treating a burn in a subject 74, which comprises reacting claimed in any one of the biological collagen in contact with the fabric burn 24-26.

74 75 method of claim wherein said burn is selected from first degree burns, second degree burns, third degree burns, infected wounds and burns burn wound impetigo.

30 76. The method of claim 68, 72 or 74, wherein the subject is a human.

Description:

说明书 胶原生物织物以及其制备和使用方法 本申请是于2002年3月26日提交的美国申请号10/106,653的部 5 分继续申请，此处以其整体引用美国申请号10/106, 653作为参考。

1. 发明领域 本发明涉及从羊膜制得的胶原膜，此处称为胶原生物织物。本发 明的胶原生物织物具有天然的未处理的羊膜的结构完整性，即天然的 三级和四级结构。本发明提供了通过对羊膜进行去细胞化而从胎盘膜 10 制备胶原生物织物的方法，优选地从具有绒毛膜和羊膜的人胎盘膜制 备。在一个优选的实施方案中，羊膜完全地进行去细胞化。本发明

Instructions collagen bio-fabric and method of making and using the present application is the US Department of Application No. 26 March 2002 filed 10 / 106,653 5 points continue to apply, reference herein in its entirety U.S. Application No. 10/106, 653 as a reference.

1. Field of the Invention The present invention relates to a collagen obtained from the amniotic membrane, referred to herein as collagen bio-fabric. Structural integrity of the present invention has a natural collagen bio-fabric untreated amniotic membrane, the natural tertiary and

的 胶原生物织物在内科和外科领域有着许多的应用，包括例如血管的修 复、血管的构建和替换、腱和韧带的替换、创伤敷裹、外科移植物、 眼科用途、缝合等等。该生物织物的优点部分地是由于其物理特性， 15 例如生物机械强度、屈曲性、可缝合性和低免疫原性，特别是当其来 源自人胎盘时。

2. 发明背景 2.1羊膜：解剖学和组织学 胎盘囊由通过疏松结締组织密切连接的两层膜组成。它们被称为 20 羊膜层和绒毛膜层（见图1)。羊膜层是两层膜中最里面的一层，其与 围绕胎儿的羊水接触，而且羊膜和羊水一起形成羊膜囊。羊膜层是无 血管的，其内表面是覆盖在基底膜上的简单的柱状上皮细胞，并且其 厚度为30-60微米。绒毛膜是囊的外层，而且其是高度细胞化的。血 管树状结构从胎盘开始，并通过绒毛膜层延伸至胎盘膜。绒毛膜层通 25 过疏松结締组织而与羊膜层分隔开，并由此联合在一起，这两层的厚 度为120-180微米。胎盘膜具有大量负载了粘多糖的胶原基质，而且 胎盘膜椐信主要用作对于正在发育的胎儿的保护性囊。这些膜还保持 了对于母体循环系统中存在的传染剂和免疫剂的屏障。胎盘膜具有主 动和被动转运。最小的分子和蛋白质能够自由地通过它们进行移动， 30 但大的蛋白质如IgM不能穿过基底层。

在以50-50%的比例与组织培养基混合的95%乙醇或者甘油中保存 胎盘膜（其为动物或人来源的）已经用于在冷冻之前保存羊膜。这些 保存剂去除了形成固缩核的胎盘组织的活力，但胶原基质核基底膜得 到了保存。有趣的是，这两种形式的保存方法还去除了移植的膜的抗 原性，和还去除了任何潜在具有毒力的试剂。保存通常在小心地将羊 膜与绒毛膜分离之后完成。羊膜具有基底层和上皮细胞的一面是光亮 5 的，而朝向绒毛膜的另一面是暗淡的。

2. 1先前的羊膜的临床应用 使用异源组织（来自其他物种的组织）而带来人兽互传疾病或者 引起种间交叉排异反应的可能潜在问题已经使得对于这些组织的期望 变小了。异源移植物或者来自同一物种的不同个体的移植物继续是人 10 移植物材料的优选的来源。用于移植的人供体组织的不足成为日益增加的问题，其促进了用于组织移植的新材料的开发。最通常的是，这 些生物天然材料的来源是不足的、难以获得的和非常昂贵的。可是， 来自人羊膜的胶原层具有所希望的在组织移植应用中有用的生物机械 特性。因此，羊膜是异源移植材料的良好来源。

15 包括羊膜和绒毛膜的胎膜已经用于外科手术，这早在1910年就得 到了证实，并且由Trelford和Trelford-Sauder于1979年进行了回 顾（见Trelford和Trelford-Sauder, 1979，Am. J. Obstet. Gynecol.

quaternary structure. The present invention provides a decellularized amniotic membrane of the placenta and membranes 10 Preparation of collagen biological fabrics, preferably from human placenta and amniotic membrane has chorionic prepared. In a preferred embodiment, the amniotic membrane were completely decellularized. Collagen biological fabric of the present invention has many areas of medical and surgical applications, including for example, vascular repair, construction, and replacement, tendon and ligament replacement blood vessels, wound dressings, surgical implants, ophthalmic use, stitching and the like. The advantages of the biological fabric is due to its physical properties, such as bio-15 mechanical strength, buckling can be sewn and low immunogenicity, especially when it is coming from the human placenta.

2 Background of the Invention 2.1 amnion: anatomy and histology placenta Nang by the two membranes closely connected by loose connective tissue composed. They are called amniotic membrane and chorion layer 20 (see FIG. 1). Amniotic membrane layer is a two innermost layer, in contact with the amniotic fluid surrounding the fetus and amnion and amniotic fluid together form the amniotic sac. Amniotic membrane is avascular, its inner surface is coated on the base film a simple columnar epithelial cells, and a thickness of 30-60 microns. Chorionic Nang is the outer layer and its cells are highly oriented. Vascular tree beginning from the placenta, and to extend through the placental chorionic membrane layer. Chorionic layer 25 through through loose connective tissue and amniotic membrane layers are separated, and thus united, the thickness of the two layers of 120 to 180 microns. Placental membranes with a large load of the collagen matrix glycosaminoglycans, and placental membranes noted in the letter is mainly used for the developing fetus protective Nang. These films are also a barrier to keep infectious agents and agents for immune system present in the maternal circulation. Placental membrane having a primary dynamic and passive transport. The smallest molecules and proteins can move freely through them, but 30 large proteins such as IgM can not cross the basal layer.

Stored in placental membranes with the ratio of 50-50 percent tissue culture media in 95 percent ethanol, or mixed glycerol (which is an animal or human origin) have been used prior to

833)。在1910年，Davis第一个报道了胎膜在皮肤移植中作为外科 材料的用途，例如在烧伤的和溃烂的皮肤上（Davis等人，1910，Johns 20 Hopkins Med. J.； 15: 307)。这些研究主要是在动物中进行的，而 人试验的结果令人失望。其后，羊膜在外科手术中的用途已经扩大了 (见例如，Stern 等人，1913，JAMA, 13: 973-4; Sabel la 等人，1913，Med. Records NY, 83: 478-80 ; de Rotth 等人，1940，Arch. Opthalmol. 23: 522-5; Sorsby 等人，1946, Br. J. Opthamlol. 30: 25 337-45)。现在其用作用于烧伤的皮肤、皮肤创伤和腿部慢性溃疡的 生物敷料，用作人造阴道的外科重建中的附属组织，和用于修复脐突 出（见例如，Trelford和Trelford-Sauder Am. J. Obstet. Gynecol. 833; Colocho 等人，1974，Arch. Surg. 109: 370-3; Faulk 等人，1980, Lancet, 1156; Prasad 等人，1986，J. Trauma, 30 26: 945-6 ; Subrahmanyan 等人，1995, J. Plastic Surg. 48: 477-8; Gruss 等人，1978，J. Can. Med. Assoc. 118: 1237-46; Ward 等人' 1984，Br. J. Plastic Surg. 37: 191-3; Dhall, J. Obstet. Gynaecol. 91: 279-82)。其还已经用于在腹部、头部 和骨盆的外科程序中防止组织粘连（Gharib等人，1996, Pediatr. Surg. Int. 11: 96-9; Rennekampff 等人，1994, Invest. Surg. 7: 187-93)。在20世纪40年代，几个作者报道了羊膜在治疗多种眼表 5 面病症中的有益作用（见例如，de Rotth等人，1940, Arch. Opthalmol. 23: 522-5; Sorsby 等人，1946, Br. J. Opthalmol. 30: 337-45; Lavery 等人，1946, Trans. Opthalmol. Soc. UK, 66: 668)。

先前已经描述了在本领域中的大量尝试，这些尝试是为了最优化 用于移植的羊膜的制备和保存（综述见例如，Dua等人，1999, Br. J. 10 Opthalmol. 83: 748-52 ("Dua") ）。各种羊腹的制备包括用盐水和抗生素混合物进行保存，和在有或没有将羊膜层与绒毛膜层分离的情 况下的醇脱水。可是，在Dua中和在上述文献中所描述的所有方法仍 具有这样的缺点，即需要羊膜的制备和保存中的改进来引导。

更近来的一段时间，已经公开了依赖于冷冻保存羊膜的方法，这 15 些冷冻保存的羊膜应用于为了矫正角膜上皮缺损所进行的组织移植外 科程序之中。见例如，U.S.专利号6，152，142和6，326，019B1 ("Tseng")。Tseng公开了这样的羊膜，即其置于基质之上，且保存 在Dulbecco氏修饰Eagle培养基和甘油的混合物之中，并于-80X：冷 冻。在其制备期间的任何时候冷冻组织的过程使得Tseng羊膜变得易 20 碎，甚至是在融解和活化步骤之后变得更加易碎。另外，融解和活化 步骤增加了操作羊膜所需的时间。此外，因为保存和制备程序中的冷

cryopreservation amnion. In addition to the formation of these preservatives vitality pyknotic nuclei placental tissue, but the basement membrane collagen matrix core was to be saved. Interestingly, these two forms of preservation methods also removes antigenic membrane transplantation, and also removes any potential virulent agent. Save usually done after carefully sheep and chorionic membrane separation. Amniotic epithelial cells having a base layer and the light side is 5, and the other side toward the chorionic bleak.

2. 1 Clinical application of previous amnion heterologous tissue (tissue from other species) which bring people pass each animal diseases that could potentially cause problems or cross-species has led to the rejection of these organizations expect smaller. Heterologous graft or graft different individuals from the same species continue to be people 10 graft material is preferably sources. Lack of human tissue for transplantation for an increasingly increasing problem, which promotes the development of new materials for tissue transplantation in. The most common is a natural biological source of these materials is inadequate, it is difficult to obtain and very expensive. However, the collagen layer derived from human amnion tissue transplantation have useful applications in the biomechanical properties desired. Therefore, a good source of amniotic membrane allograft material.

15 comprising amnion and chorion membranes have been used in surgery, which as early as 1910 has been confirmed, and by the Trelford and Trelford-Sauder reviewed (see Trelford and Trelford-Sauder in 1979, 1979, Am. J. Obstet. Gynecol. 833). In 1910, Davis first reported the transplantation of skin membranes in use as a surgical material, such as burns and ulcers on the skin (Davis et al., 1910, Johns 20 Hopkins Med J.; 15:. 307). These studies are mainly conducted in animals, human trials and the results have been disappointing. Thereafter, the amniotic membrane in surgery use has expanded (see, for example, Stern et al., 1913, JAMA, 13: 973-4; Sabel la et al., 1913, Med Records NY, 83: 478-80; de.. Rotth et al., 1940, Arch Opthalmol 23: 522-5; Sorsby et al., 1946, Br J. Opthamlol 30:.. 25 337-45).. Now it is used for burns of the skin, skin biological dressing wounds and chronic leg ulcers, surgical reconstruction is used as an artificial vagina in the affiliated organizations, and for the repair of

冻步骤所引起的Tseng羊膜的易碎性，因此需要结构支持物或背衬来 确保Tseng羊膜在贮存期间的结构完整性。这就带来了将保存的羊膜 与背衬分离开的附加困难，该羊膜由于其易碎性而难以操作和完整地 25 分离。因此将羊膜与背衬分离增加了膜破裂的可能性，和增加了活化 羊膜所需的时间长度，这一时间使得能够在进行外科程序之前让活化 溶液完全浸透冷冻的羊膜，从而导致增加了外科手术系列过程中的准 备时间。贮存和运输还由于需要-80X：冷冻而变得复杂。最后，Tseng 的膜一般是不去细胞化的；因此，如此制备的羊膜通常是不透明的， 30 而且不具有一致的结构组成。

更近来的一段时间，Yui等人（U.S.专利号5，618，312 ("'312 专利")）描述了从组织膜致密层中制备胶原层。虽然所描述的材料主 要是胶原，但是其由于其处理方法而太脆弱，以致需要单独的交联步 骤来使其变得足够结实以用于医学用途，例如可用于缝合。在，312专 利中描述的一种交联方法采用高热处理，优选地于100-110X：，这据 信对于肢原的天然构象产生不利影响。Yui等人（U.S.专利号 5 5, 876，451)描述了类似的来源自胎盘的胶原材料。可是，这种材料在 制备期间作为去细胞化步骤的一部分而用蛋白酶进行处理，这很可能 导致基质组分的天然构象的破坏和/或瓦解；因此，结果所得的胶原不 能保持其天然构象。

因此，在本领域中继续存在需要来获得经改良的羊膜以用于医 10 疗、诊断和美容应用，其具有经改善的结构特性。本发明提供了这样 一种含有胶原的生物织物，其不同于以前的基于胶原的生物织物而保 持了天然的胶原四级结构。因此，本发明的生物织物容易制备、容易 使用，和其强度足够用于内科和外科目的，并提供了用于伤口愈合的 优良基质。

15 3. 发明概述 本发明部分地涉及发明者发现一种新型方法来从胎盘（优选为人 胎盘）中制备胶原生物织物，这导致获得具有改善的物理和生物物理 特性的新型胶原生物织物。该制备方法优选地包括尽量减少羊膜的操 作。本发明的胶原生物织物由于其处理方法而与现有技术中所描述的 20 那些不同，其具有完整的天然三级和四级结构。本发明还提供了来源 自胎盘的羊膜或生物织物，其具有优良的特性，即增强的抗拉强度、 可缝合性和导致减少宿主-移植物排异的减少的免疫源性。本发明还提 供了来源自胎盘的羊膜或生物织物，其能够作为脱水的层进行贮存， 而不需冷冻或深低温保藏。优选地，来源自胎盘的羊膜来自人胎盘以 25 在人患者中使用。可是，可以用来自多种动物物种的胎盘而使用同样 的方法以在患病动物中进行兽医学应用。

omphalocele (see, for example, Trelford and Trelford-Sauder Am. J.. Obstet Gynecol 833; Colocho et al., 1974, Arch Surg 109:.. 370-3; Faulk et al., 1980, Lancet, 1156; Prasad et al., 1986, J Trauma, 30 26:.. 945-6;. Subrahmanyan et al., 1995, J. Plastic Surg 48: 477-8; Gruss et al., 1978, J Can Med Assoc 118:... 1237-46; Ward et al., '1984, Br J. Plastic Surg.. 37: 191-3; Dhall, J. Obstet Gynaecol 91:.. 279-82). It also has been used in the abdomen, head and pelvic surgical procedures to prevent tissue adhesion (Gharib et al., 1996, Pediatr Surg Int 11: 96-9; Rennekampff et al., 1994, Invest Surg 7...: 187-93). In the 1940s, several authors reported the beneficial effects of amniotic membrane in the treatment of various ocular surface disorders in Table 5 (see, for example, de Rotth et al., 1940, Arch Opthalmol 23:.. 522-5; Sorsby et al.,.. 1946, Br J. Opthalmol 30: 337-45; Lavery et al., 1946, Trans Opthalmol Soc UK, 66:... 668). previously been described in the art in numerous attempts, these attempts to optimize for transplantation of amniotic membrane preparation and preservation (for review, see for example, Dua et al., 1999, Br J. 10 Opthalmol 83..: 748-52 (" Dua ")). preparation of various sheep abdominal include antibiotic mixture with brine and save, and in the presence or absence of the amniotic membrane and chorionic layer was separated case alcohol dehydration. However, in Dua All the above methods described in the literature, and still has the disadvantage of requiring the preparation and preservation of amniotic improvements to boot.

More recently a period of time, it has been disclosed that rely on cryopreservation of amniotic membrane method, 15 of these organizations that cryopreserved amniotic membrane used in order to correct the corneal epithelial defects among conducted transplant surgical procedure. See, for example, U.S. Patent Nos. 6,152,142 and 6,326,019B1 ("Tseng"). Tseng discloses amnion, i.e., placed over the substrate, and stored in modified Eagle's medium and Dulbecco's being a mixture of glycerol and at -80X: freezing. Becomes more friable at any time during its preparation frozen tissue during such Tseng amniotic become, 20 pieces, and even in the melt after the activation step. In addition, the melting and activation steps to increase the time required for the operation of the amnion. In addition, since the preparation of the program and save the frozen steps caused Tseng amniotic membrane fragility, requiring structural support or backing to ensure Tseng

本发明提供了制备胶原生物织物的方法，包括提供胎盘优选为人 胎盘，分离羊膜层和绒毛膜层，和将羊膜进行去细胞化，并同时保留 下面的细胞外基质的结构。该方法此外还需要洗涤和干燥经去细胞化 30 的膜。该方法产生脱水的、去细胞化的生物织物，其能够在无菌的贮 存条件下于室温保持稳定，而且随后进行再水化并移植入受试者中。

在一个特殊的实施方案中，胎盘来自经历了剖腹产术分娩或者自 然分娩的女性人类。在优选的实施方案中，使用对于本领域熟练技术 人员已知的常用的血清学和细菌学测定来确定胎盘是否来自没有传染病的供体。在一个特殊的实施方案中，胎盘经过测试而没有至少一种 传染病。在另一个实施方案中，胎盘的来源是已知的，包括供体的医 5 疗史、血型、免疫学数据和基因型特征。虽然胎盘可以来自任何哺乳 动物，但供体优选为女性人类。在一些实施方案中，在将羊膜和绒毛 膜分离开之前，用本领域熟练技术人员已知的常用方法来去除胎盘中 的血。

根据本发明的方法将羊膜进行去细胞化，这包括从羊膜上去除所 10 有可见的细胞材料和细胞碎片，例如从羊膜的母体侧和从羊膜的胎儿 侧。羊膜的去细胞化不应当导致羊膜结构完整性的破坏或者改变生物 化学组成。因此，根据本发明的方法进行羊膜的去细胞化不会要求在 制备羊膜过程中的任何一点上进行冷冻或者将膜与蛋白酶进行接触。

羊膜优选地用含有弱去污剂的溶液进行去细胞化，例如含有0. 01-1. 0% 15 —水合脱氧胆酸钠、非离子去污剂、Triton X-100、阴离子去污剂或 十二烷基硫酸钠或者其组合的溶液。

一旦根据本发明的方法将羊膜进行去细胞化，膜就可以进行洗涤 和干燥，优选地用低热在真空中进行。

在一些实施方案中，本发明提供了含有脱水的、去细胞化的且无 20 基质的羊膜的胶原生物织物，因此膜具有天然的三级和四级结构。在 其他实施方案中，本发明提供了含有去细胞化的且无基质的羊膜的胶 原生物织物，其含有胶原、弹性蛋白和纤连蛋白。在其他实施方案中， 本发明提供了含有脱水的、去细胞化的、均一的、半透明的和无基质 的羊膜的胶原生物织物，并且规定羊膜从未与蛋白酶接触。

25 在一些实施方案中，生物织物此外还含有一种或多种生物分子， 例如治疗剂，包括抗生素、激素、生长因子、抗肿瘤剂、抗真菌剂、 抗病毒剂、疼痛药物、抗组胺剂、抗炎剂、抗感染剂、伤口愈合

amniotic membrane structural integrity during storage. This brings the added difficulty of preserved amniotic membrane with the backing separated from the amniotic membrane due to its fragility and difficult to handle and complete 25 separate. Therefore, the amniotic membrane and increases the possibility of backing the separation membrane rupture, and increases the length of time required to activate the amnion, so this time before it can be activated during a surgical procedure so that the solution is completely saturated frozen amniotic membrane, resulting in an increase of Surgery preparation time series during surgery. Storage and transport are also due to the need -80X: Frozen complicated. Finally, Tseng membranes of cells are generally not; therefore, amniotic membrane thus prepared are usually opaque, and 30 do not have a consistent structure.

More recently a period of time, Yui et al. (U.S. Patent No. 5,618,312 (" '312 patent")) describes the preparation of a dense layer of tissue membranes from collagen layer. Although the collagen material mainly described, but the treatment method because of its too fragile, that may require a separate cross-linking steps make it strong enough to be used for medical purposes, for example, it can be used for suturing. A cross-linking method, described in the 312 patent with high heat treatment, preferably in 100-110X:, which are believed to limb original native conformation adversely affected. Yui et al. (U.S. Patent No. 55, 876,451) describes a collagen-like material sources from the placenta. However, this material during the preparation as part of a decellularized step and with protease treatment which is likely to result in the destruction matrix component native conformation and / or collapse; therefore, the results obtained collagen is not able to maintain its native conformation.

Thus, there is a need to continue to access to improved amniotic membrane in the art for use in the medical 10 treatment, diagnostic and cosmetic applications, structural characteristics have been improved. The present invention provides such a biological fabric containing collagen, which differs from previous collagen-based bio-fabric and maintain the natural collagen quaternary structure. Thus, the fabric of the present invention is the biological readily prepared, easy to use, and intensity sufficient for medical and surgical

剂、伤口封闭剂、细胞吸引剂和支架试剂等等，但并不局限于此。在一个特殊的实施方案中，胶原生物织物可以用一种或多种生长因子进行浸30渍，例如成纤维细胞生长因子、上皮细胞生长因子等等。生物织物还可以用一种或多种小分子进行浸渍，包括小有机分子如特殊生物化学过程的特异性抑制剂，例如膜受体抑制剂、激酶抑制剂、生长抑制剂、抗癌药物、抗生素等等，但并不局限于此。在一些实施方案中，胶原生物织物在生产期间或者在用于外科手术的准备期间用生物分子进行浸渍，这取决于其想要的用途。

在一些实施方案中，本发明包括含有至少两层本发明的生物织物5的层压制品，和包括制备该层压制品的方法。在其他实施方案中，本发明包括依赖于想要的用途而将该层压制品定形成复杂的三维支架，包括片层、纤维、球、管，但并不局限于此。

在一个实施方案中，本发明包括制备羊膜层压制品的方法，其包括提供含有羊膜和绒毛膜的胎盘；分离羊膜和绒毛膜；和将羊膜进行10去细胞化。在另一个实施方案中，该方法此外还包括洗涤去细胞化的羊膜至少一次；将至少两层去细胞化的羊膜以相互接触的方式层叠在一起，从而形成羊膜层压制品；和干燥去细胞化的羊膜层压制品。可选择地，在另一个实施方案中，用于制备羊膜层压制品的方法包括干燥至少两层根据本发明制备得到的羊膜，和将至少两层羊膜以相互接15触的方式层叠在一起，从而形成羊膜层压制品。

在一些实施方案中，根据本发明的方法生产出的羊膜层可以在存在粘合剂的条件下以相互接触的方式放置在一起，以形成羊膜层压制品。根据本发明的方法和组合物所使用的粘合剂可以为任何本领域熟练技术人员已知的生物胶合剂，优选为生物相容的胶合剂，其包括天20然胶合剂如纤连蛋白、血纤蛋白以及合成的胶合剂，但并不局限于此。

在其他实施方案中，将根据本发明的方法制备得到的羊膜层相互进行交联以形成羊膜层压制品。任何本领域熟练技术人员已知的交联试剂和方法在本发明的范围之内，包括化学交联、肽交联、UV交联、放射交联、纤连蛋白交联、血纤蛋白原交联、水凝胶交联，但并不局限于25此。在其他实施方案中，根据本发明的方法生产出的羊膜层压制品不含有粘合剂。

在一些实施方案中，本发明包括使用胶原生物织物作为外科移植物。在一个特殊的实施方案中，本发明包括于在受试者（优选为人）中进行的外科程序之中使用外科移植物，这包括将移植物直接置于外30科部位。

purposes, and to provide excellent matrix for wound healing.

15 3. Summary of the Invention The present invention relates in part inventors have discovered a novel way to from placenta (preferably a human placenta) prepared in biological collagen fabric, which results in new collagen to obtain a fabric having physical and biological biophysical properties of improved. The preparation method preferably includes minimizing amniotic operation. Collagen biological fabric of the present invention because of its treatment method and 20 are different from those described in the prior art, which has the complete native tertiary and quaternary structure. The present invention also provides a source from placenta or amniotic biological fabric, which has excellent properties, i.e., tensile strength increased, and stitching may lead to a reduction of host - graft rejection reduced immunogenicity. The present invention also provides a source from the placenta or amniotic biological fabric, which can be stored as the dehydrating layer, without freezing or cryopreservation. Preferably, the source from the placenta amnion from human placenta to 25 for use in human patients. However, you can use the placenta from a variety of animal species and using the same methods for veterinary use in sick animals.

The present invention provides a method for preparing biological collagen fabrics, including preferably a human placenta placenta, and amniotic membrane and chorion layer separation, and the decellularized amniotic membrane, while retaining the following structure of the extracellular matrix. This method also requires washing and drying the decellularized 30 film. The method of producing dehydrated, decellularized biological fabric, which can remain stable at room temperature Storage under sterile conditions, and subsequently rehydrated, and transplanted into a subject.

In a particular embodiment, the placenta from cesarean delivery experienced a natural childbirth or female human. In a preferred embodiment, the use of the skilled artisan commonly used bacteriological and serological assay to determine whether the placenta from no infectious disease donor. In a particular embodiment, the placenta is tested without at least a contagious disease. In another embodiment, the source of the placenta

本发明提供了胶原生物织物、羊膜层压制品或者进一步含有一种 或多种水凝胶组合物的三维支架，和提供了制备这些材料的方法。水 凝胶组合物可以含有聚合物，包括聚乙烯醇、聚乙二醇、透明质酸以及其衍生物和类似物，但并不局限于此。

在一些实施方案中，可以进一步在本发明的胶原生物织物、层压 制品、三维支架或者其水凝胶组合物上移植细胞，例如干细胞、分化 5 的成熟细胞、祖细胞等等，优选为人细胞，从而这些细胞是均一的和 汇合的。

本发明提供了高水平生产胶原生物织物、其层压制品、其三维支 架和其水凝胶组合物的方法，特别是商业规模的生产，但并不局限于 此。本发明解决了在生产大量的用于临床试验和商业销售的羊膜中的 10 困难。

本发明包括含有本发明的胶原生物织物的组合物，其适合于药物 递送；组织工程；泌尿学相关用途，例如尿失禁的矫正；眼的用途， 例如用于治疗眼表面病症和作为眼外科移植物；血管的用途，例如血管的修复、血管的构建和替换；心脏病学的用途，例如在构建有病的 15 瓣膜中作为假体装置；神经元相关用途，例如修复受损伤的神经（尤 其是切断的外周神经）、作为硬脑膜的替代物和作为围绕神经吻合的 假体；骨相关用途，例如用于处理矫形缺陷、作为骨替代物；皮肤病 学的用途，例如用于治疗伤口（外部的和内部的）、急性和慢性伤口、 先天性伤口和烧伤；用于处理皮肤状况，例如皮肤损伤、老化的皮肤、 20 皱纹、细纹、变薄、降低的皮肤弹性、粗糙的皮肤和阳光损害的皮肤； 作为创伤敷料；和用于治疗伤口感染。

在一个特殊的实施方案中，本发明包括用于在受试者中治疗和/或 预防眼相关疾病或病症例如眼表面疾病的方法，其包括使用本发明的 胶原生物织物，例如通过将生物织物作为外科移植物置于受试者的有 25 病的角膜表面上。在另一个实施方案中，本发明包括在受试者中处理 和/或预防皮肤状况，其包括使用本发明的生物织物，例如通过将生物 织物与皮肤接触。在另一个实施方案中，本发明包括在受试者中治疗 创伤和/或烧伤，其包括将本发明的生物织物与创伤和/或烧伤处接 触。在另一个特殊的实施方案中，本发明包括在受试者（优选为人） 30 中矫正尿失禁的方法，其包括使用本发明的胶原生物织物作为植入 物。

在其他实施方案中，本发明包括向受试者递送治疗剂的方法，其 包括将本发明的胶原生物织物与受试者接触。此外本发明还包括向受 试者递送细胞，其

are known, including five therapy Medical history of the donor, blood type, immunological data and genotype characteristics. Although the placenta may be from any mammal, but preferably female human donor. In some embodiments, prior to separating the amniotic membrane and fluff, with skilled technical personnel known to commonly used methods to remove the placenta blood.

The method according to the present invention will be decellularized amniotic membrane, which includes removing the amniotic membrane from the 10 visible cellular material and cellular debris, such as the fetus from the maternal side of the amniotic membrane side and from the amnion. Amnion decellularization should not lead to the destruction of the structural integrity of the amniotic modified organisms or chemical composition. Therefore, the amniotic membrane according to the method of the present invention does not require any of the cells to the point in the preparation process of the amniotic membrane was frozen or in contact with a protease. amnion preferably made decellularized weak detergent solutions containing, for example, contain from 0 percent 0. 01-1 15. - Hydrated sodium deoxycholate, non-ionic detergent, Triton X-100, to anionic or detergent sodium dodecyl sulfate solution or a combination thereof.

Once the method according to the present invention will be decellularized amniotic membrane can be washed and dried, preferably with low heat in a vacuum.

In some embodiments, the present invention provides a composition containing dehydrated, decellularized matrix 20 and no amnion collagen bio-fabric, so the film has a natural tertiary and quaternary structure. In other embodiments, the present invention provides a collagen-containing biological fabric decellularized matrix and no amnion, which contains collagen, elastin and fibronectin. In other embodiments, the present invention provides a composition containing dehydrated, decellularized, uniform, translucent and non-biological matrix amnion collagen fabric, and has never been in contact with the amniotic provisions protease.

25 In some embodiments, the biological fabric also contains one or more biological molecules, such as therapeutic agents, including antibiotics, hormones, growth factors, anti-tumor agents,

包括例如在组织工程中在本发明的胶原生物织物或层压制品上移植活细胞。

本发明提供了用于外科程序的并含有本发明的生物织物的外科移 5 植物。该外科移植物可以应用于受试者（优选为人）的内部或外部部 位。

4. 附图简述 图1人胎盘的绒毛膜和羊膜 图2胶原生物织物的显微照片 10 A.处理前 B.处理后 图3胶原生物织物。举例说明了本发明的胶原生物织物，其具有 带有绸纹图案的均一的半透明表面。

图4网架和正在那里干燥的生物织物 15 5. 发明详述 本发明提供了来源自哺乳动物（优选为人）的胎盘的胶原膜或生 物织物。制备胶原生物织物，以在终产品中保持天然的胶原构象，即 天然的三级和四级构象。除了胶原生物织物之外，本发明还提供了制造胶原生物织物的方法，和在医学环境中使用该生物织物的方法。

20 本发明提供了含有脱水的、去细胞化的且无基质的羊膜的胶原生 物织物，因此羊膜具有天然的三级和四级结构。在一些实施方案中， 本发明提供了去细胞化的且无基质的胶原生物织物，其含有胶原、弹 性蛋白和纤连蛋白。

在一些实施方案中，本发明提供了含有本发明的胶原生物织物的 25 羊膜层压制品。根据本发明的方法制备得到的羊膜层压制品含有至少 两层胶原生物织物，这些胶原生物织物以相互接触的方式放置在一起 以形成羊膜层压制品。在其他实施方案中，本发明提供了含有本发明 的胶原生物织物的三维支架，例如管。在其他实施方案中，本发明提 供了本发明的胶原生物织物、其层压制品或者进一步含有水凝胶组合 30 物的其三维支架。

因此，本发明提供了多种形式和构造的胶原生物织物，包括层压 制品、三维支架和水凝胶组合物，但并不局限于此。本发明提供了本 发明组合物的任何医学上有用的形式。不管特殊的形式或构造，本发明的组合物可以进一步含有一种或多种生物分子，优选为治疗剂。正 如此处所详细描述的，含有生物分子的本发明的组合物在内科领域中 有着许多用途。在一些实施方案中，本发明包括在本发明的组合物上 5 移植活细胞，从而这些细胞是均一的和汇合的。移植了细胞的本发明 的组合物在内科和牙科领域中有着许多用途，例如用于组织工程目 的。

本发明还涉及制备胶原生物织物、其层压制品、其三维支架或水 凝胶组合物的方法。在一个特殊的实施方案中，本发明提供了从具有 10 羊膜和绒毛膜的胎盘制备胶原生物织物的方法，包括：分离羊膜

antifungal agents, antiviral agents, pain medications, antihistamines, anti-inflammatory agents, anti-infective agents, wound healing agents, wound sealant, cellular attractant and bracket reagent, etc., but is not limited thereto. In a particular embodiment, the collagen may be biological fabric with one or more growth factors dip 30 stains, e.g., fibroblast growth factor, epidermal growth factor, and so on. Biological fabric may also be treated with one or more small molecule impregnation, including small organic molecules such as specific inhibitors of particular biochemical processes, e.g., membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, and so on, but it is not limited thereto. In some embodiments, the biological collagen fabric during production or during preparation for surgery is impregnated with a biological molecule, depending on its intended use.

In some embodiments, the present invention includes at least two layers of the present invention, the biological fabric laminate 5, and includes a method for producing the laminate contains. In other embodiments, the present invention includes dependent on the intended use and the laminate is shaped into a complex three-dimensional scaffold, including sheets, fibers, spheres, tubes, but is not limited thereto.

In one embodiment, the present invention includes a method for preparing amniotic laminate, which includes providing the placenta and amniotic containing chorion; amnion and chorion separation; and the decellularized amniotic membrane 10. In another embodiment, the method also includes washing decellularized amniotic membrane at least once; at least two layers of decellularized amniotic membrane in contact with each other stacked together to form the amniotic membrane laminate; and dried acellular of amniotic membrane laminate. Methods Alternatively, in another embodiment, for the preparation of dried amniotic membrane laminate comprises at least two layers prepared according to the present invention obtained amnion, amniotic membrane and the at least two mutually connected contact 15 is laminated together, thereby forming a laminate amnion.

In some embodiments, produced according to the method of the present invention, amnion layer may be present in the binder under conditions to be

层和 绒毛膜层；和将羊膜进行去细胞化，以致羊膜不与酶例如蛋白酶接触。

在其他实施方案中，该方法此外还需要洗涤和干燥经去细胞化的羊 膜。在另一个特殊的实施方案中，本发明提供了从具有羊膜和绒毛膜 的胎盘制备羊膜层压制品的方法，包括：分离羊膜层和绒毛膜层；将 15 羊膜进行去细胞化；和将至少两层去细胞化的羊膜以相互接触的方式 层叠在一起，从而形成羊膜层压制品。在一个特殊的实施方案中，去 细胞化的羊膜在层叠之前进行干燥。在另一个特殊的实施方案中，去 细胞化的羊膜在层叠之后进行干燥，以致于至少两层去细胞化的羊膜 相互接触。

20 本发明提供了在内科、牙科和外科环境中使用本发明的胶原生物 织物、其层压制品、其三维支架或其水凝胶组合物的方法。其实，期 望本发明的组合物相对于本领域中已知的其他生物材料具有增强的治 疗和临床效用。在一些实施方案中，本发明提供了使用本发明的组合 物在受试者中治疗和/或预防眼相关疾病或病症的方法。在一个特殊的 25 实施方案中，本发明提供了在受试者中治疗和/或预防眼相关疾病或病 症的方法，其包括将生物织物置于受试者的患病的眼表面上。

在其他实施方案中，本发明提供了用本发明的组合物在受试者（优 选为人）中处理和/或预防皮肤状况的方法。在一个特殊的实施方案 中，该方法包括将组合物与受试者的皮肤接触。在另一个特殊的实施 30 方案中，将组合物直接置于受试者的皮肤表面上，该皮肤表面是皮肤 状况的部位。

本发明还提供了在受试者（优选为人）中治疗创伤或烧伤的方法， 其包括在创伤或烧伤的部位接触组合物。

在其他实施方案中，本发明提供了在外科程序中使用本发明的组 合物（例如胶原生物织物、羊膜层压制品），例如在眼外科、心血管 外科、牙周外科、神经外科、牙外科和矫形外科。

5 本发明提供了使用本发明的组合物来向受试者（优选为人）递送 生物分子（优选为治疗剂）的方法。本发明还包括使用本发明的组合 物来向受试者（优选为人）递送细胞的方法，其中已经进一步在组合 物上移植了细胞。

5. 1股原生物织物 10 本发明提供了胶原羊膜，此处称为胶原生物织物。本发明的胶原 生物织物保持了天然的、未处理的羊膜的结构完整性，即在其组成中 保持了结构蛋白（例如胶原、弹性蛋白或许还有纤连蛋白）的三级和 四级结构。因此，本发明的胶

placed in contact with each other together to form a laminate of amniotic membrane. The adhesive can be proficient methods and compositions of the present invention is used in any known to those skilled in the biological glue, preferably a biocompatible adhesive, including a 20-day natural adhesive such as fibronectin, and synthetic fibrin glue, but not limited thereto.

In other embodiments, the layer of each other according to amniotic method of the present invention prepared by the cross-linked to form amniotic membrane laminate. Any cross-linking reagents and methods known to a person skilled in the art within the scope of the present invention, including chemical cross-linking, cross-linking peptide, UV crosslinking, radiation crosslinking, crosslinking fibronectin, fibrinogen post linking, cross-linked hydrogel, but 25 is not limited to this. In other embodiments, produced according to the method of the present invention is not rounded amniotic laminate adhesive.

In some embodiments, the present invention includes the use of collagen as a biological fabric surgical graft. In one particular embodiment, the present invention being included in a surgical procedure in a subject (preferably a human) performed using a surgical graft, including graft placed directly in the outer parts of the 30 families.

The present invention provides a collagen bio-textiles, amniotic membrane laminate or further comprising one or more hydrogel composition of scaffolds, and provides methods of preparing these materials. The hydrogel composition may contain polymers including polyvinyl alcohol, polyethylene glycol, hyaluronic acid and derivatives and the like, but is not limited thereto.

In some embodiments, the collagen can be further biological fabric of the present invention, laminate, scaffolds or a hydrogel composition of the transplanted cells, such as stem cells, differentiation and maturation of cells, progenitor cells 5, etc., preferably a human cell, so that these cells are uniform and confluent.

The present invention provides a high-level production of collagen bio-fabric, a laminate thereof, and methods of three-dimensional scaffold which hydrogel composition, especially the commercial-scale production, but is not limited thereto. The present invention solves the production of large amounts of amniotic

原生物织物由与天然的或未处理的羊膜 一样的结构蛋白所组成。生产羊膜的现有技术方法需要使用蛋白酶或 15 高热处理，因此这些膜不能在它们的组成中保持结构蛋白的三级和四 级结构。

在一个特殊的实施方案中，本发明提供了脱水的、去细胞化的且 无基质的（即无过滤背村）羊膜，因此羊膜具有天然的三级和四级结 构（见图2A和B)。本发明的脱水的、去细胞化的羊膜是均一的（即 20 最少至没有细胞材料）、半透明的生物织物，其具有图3中显示的外 观，其具有去细胞化的基质的左手三股螺旋a螺旋片（见例如， Molecular Biology of the Cell, 1989，Alberts 等人（编者），Garland Publishing公司，New York, NY;此处以其整体进行引用 作为参考）。

25 本发明的胶原生物织物可以来源自任何哺乳动物的羊膜，例如 马、牛、猪或狭鼻类动物来源，但是最优选的是来源自人胎盘。在一 个优选的实施方案中，本发明的生物织物在其组成中具有胶原材料的天然的三级和四级结构。

与现有技术中所描述的那些不同，本发明的胶原生物织物以最小 30 限度进行操作，即本发明的胶原生物织物进行至多一个化学或生物处 理或者操作，例如在弱去污剂中进行去细胞化。在此处使用时，“以 最小限度进行操作”是指在制备胶原生物织物期间的任何步骤，本发 明的羊膜没有进行酶处理例如蛋白酶处理、高热处理、强烈的化学处 理、暴露于强去污剂或酸。作为去细胞化步骤的一部分，羊膜的蛋白 酶处理会损害生物织物的结构完整性，例如影响胶原材料的三级和/或 四级结构。与此不同，在制备本发明生物织物中的羊膜的最小限度的 5 操作导致获得相对于现有技术中的产品来说具有增强的机械强度的产 品和半透明的产品。

本发明的去细胞化的且无基质的胶原生物织物含有胶原（包括I 型、IV型和II型胶原，但并不局限于此）以及纤连蛋白和弹性蛋白。

这种联合部分地是造成根据此处描述的方法制备得到的本发明生物织 10 物具有增强的机械强度的原因。作为最小限度的处理和操作的结果， 本发明的胶原生物织物保持了天然膜的组成。胶原是主要的脊推动物 结构材料，其存在于主要具有机械功能的组织中。胶原分子由三条称 为a链的多肽链（每条的长度为大约1000个氨基酸）构成，这三条链 如在三股绳中一样相互缠绕在一起，从而形成规则的左手超螺旋。已 15 经鉴定了至少胶原的19种类型，它们都具有同样的三维组构（见例如， Lee 等人，2001, International J. of Pharmaceutics, 221: 1-22)。本发明的羊膜在形式、生物机械和结构特征

membrane used in clinical trials and commercial sale of 10 difficulties.

The present invention comprises a collagen-containing biological fabric of the present invention is a composition, which is suitable for drug delivery; tissue engineering; urology-related purposes, such as urinary incontinence correction; ophthalmic uses, such as for the treatment of ocular surface disorders and as ophthalmic surgical graft; vascular uses, such as vascular repair, construction and replacement vascular; cardiology uses, such as in the construction of the sick valve 15 as a prosthetic device; neuron-related purposes, such as repairing damaged nerve (especially severed peripheral nerves), and as a dura substitute as around nerve anastomosis prosthesis; bone-related purposes, such as for treating orthopedic defects, as bone substitutes; dermatological uses, for example for treatment of wounds (external and internal), acute and chronic wounds, burn wounds and congenital; for treating skin conditions, e.g., skin damage, skin aging, 20 wrinkles, fine lines, thinning, reduced skin elasticity, rough skin and sun damaged skin; as a wound dressing; and for the treatment of infected wounds.

In one particular embodiment, the present invention includes a treatment in a subject and / or prevention of eye-related diseases or disorders such methods ocular surface disease, including the use of the present invention, collagen bio-textiles, for example, by the bio-textiles as surgical implants placed 25 subjects on the corneal surface disease. In another embodiment, the present invention comprises treating in a subject and / or prophylaxis of skin conditions, comprising a fabric of the present invention is the use of biological, e.g., by biological fabric contact with the skin. In another embodiment, the present invention comprises the treatment of wounds and / or burn in a subject, comprising the biological fabric of the present invention and the trauma and / or burn in contact. In another particular embodiment, the present invention includes a subject (preferably a human) in 30 to correct urinary incontinence, which comprises the use of biological collagen fabric of the invention as an implant.

In other embodiments, the present invention includes the delivery of therapeutic agents to a subject, comprising biological collagen in contact with the fabric of the present invention to the

上比现有技术中所报 道的和发明者已知的那些要优良，这部分地是基于如此处所述发现了 20 制备羊膜的新型方法和人胎盘的受控和充足的来源（见5. 2节）。

特别地，与一种或多种现有技术的羊膜相比，本发明的胶原生物 织物具有一个或多个下列的特征：增强的抗拉强度；优良的可缝合性； 减小的免疫原性，这导致减小宿主-移植物排异反应；容易贮存和运 输，而不需冷冻或深低温保藏；在制备之后最小限度需要操作和活化， 25 即再水化、使用前的程序；和能够于室温贮存延长的一段时间，而同 时保持结构和功能的完整性。表1概括了本发明的生物织物与现有技 术中的那些相比时所得的优点。

在可选择的实施方案中，本发明提供了含有脱水的、去细胞化的 且无基质的绒毛膜（优选为人绒毛膜）的胶原生物织物。期望含有绒 30 毛膜的胶原生物织物会具有与含有羊膜的本发明胶原生物织物相当的 特性。本发明提供了舍有绒毛膜的胶原生物织物的所有医学有用形 式，包括层压制品、三维支架和水凝胶组合物，但并不局限于此。

表1.本发明的生物织物与传统的羊膜制3 刊的比较

特征	本发明的胶原生物织物	传统的羊膜
血清学测试	完全：抗体筛选（ATY)；丙氨酸 转氨酶筛选（ALT)；肝炎核心抗 体（核酸和ELISA);乙型肝炎表 面抗原（核酸和ELISA)；丙型肝 炎病毒抗体（核酸和ELISA)； HIV-I 和 HIV-2 ； HTLV-I 和 HTLV-2;梅毒测试(RPR) ； CMV 抗体测试；丙型肝炎和HIV测试 (核酸测试）	不完全：无CMV测试
保存	干燥：用低热和真空进行脱水；	不需冷冻机或冷藏设备；
架式贮 存	冷冻：在培养基中贮 存；	需要干冰运输和冷 冻机/冷藏设备
外科的准 备	几分钟：在几分钟之内准备；	直 接在外科部位上水化；例如在眼 上用盐水滴进行水化；
无需融 化、浸泡或漂洗	长时间：需要大量的准 备/融化/浸泡时间；	必 须去除过滤背衬
外科的操 作	简单：可以在干燥的同时进行修 整；	和应用于外科部位，然后在 该部位上水化
繁重：必须移去滤纸；		在外科程序期间倾向 于“团成球状”；
难于	在外科上进行操作	细胞性
去细胞化的：	最少至没有上皮细 胞；	最少至没有细胞碎片；
较快	的重建	含有死细胞：存在死细 胞；
临床证明表明了较	长的愈合时间/较高的	排异率（无对照的证 明）
基质	无：无基质（例如无过滤背衬）	有：支撑在硝化纤维素 /滤纸之上
灭菌（可 选的）	有：电子束辐射；至少18 kGy;	增加了组织安全性的保证
无：在含有抗生素和甘 油（有毒）的培养基中	贮存；	没有正式的最后
灭菌	组织透明 度	半透明的：光学透明
不透明的：	乳白色或混 浊的外观	在一个优选的实施方案中，本发明的胶原生物织物是半透明的， 即是光学透明的。在另

subject. Furthermore, the invention also includes a cell delivery to a subject, including, for example in tissue engineering in the biological collagen fabric or laminate of the present invention transplant living cells.

The present invention provides a method for surgical procedures and containing biological fabric of the present invention is a surgical shift 5 plants. The surgical implant may be applied to a subject (preferably a human) portion of an internal or external location.

4. 1 is a graph of human chorionic placenta and amniotic 2 collagen bio-microscopic photograph of the fabric before treatment 10 A. B. Figure 3 collagen processing biological fabric. It illustrates the biological collagen fabric of the invention, which has a translucent surface with uniform patterns on silk.

4 grid and there are biological drying fabric 15 5. DETAILED DESCRIPTION The present invention provides a source from a mammal (preferably a human) or biological collagen membrane fabric placenta. Preparation of collagen bio-textiles, in order to maintain the natural collagen in the final product conformation, namely natural tertiary and quaternary conformations. In addition to the biological collagen fabric, the present invention also provides a method of manufacturing biological collagen fabric, and methods of using the bio-textiles in the medical environment.

20 The present invention provides a composition containing dehydrated, decellularized collagen and bio-fabric matrix-free amniotic membrane, and therefore has a natural amniotic tertiary and quaternary structure. In some embodiments, the present invention provides a decellularized matrix of collagen and no biological fabric, its homes collagen, elastin and fibronectin.

In some embodiments, the present invention provides a collagen comprising the present invention biological amniotic membrane laminate fabric 25. The method according to the present invention is obtained by preparing a laminate comprising at least two layers of amnion collagen biological fabrics, these fabrics collagen organisms placed in contact with each other together to form a laminate amnion. In other embodiments, the present invention provides a three-dimensional collagen scaffold containing biological fabric of the present invention, such as

一个优选的实施方案中，本发明的胶原生物织 物是薄的和轻的。在一个特殊的实施方案中，脱水的本发明的胶原生 5 物织物为0.3-0.6 mg/cm2。在一个特殊的实施方案中，本发明的胶原 生物织物的厚度为至少30微米。在另一个特殊的实施方案中，本发明 的胶原生物织物厚度为大约10-40微米。

本发明包括使用多种构造的本发明的胶原生物织物，例如插入 物、防护物。一般地，生物织物可以定形成任何医学上有用的形式。

10 例如，胶原生物织物可以设定为以其整体进行使用，即使用来自完整 胎盘的胶原生物织物。可选择地，胶原生物织物可以切成条、片或卷， 或者可以织成线。在另一个实施方案中，作为单厚度的片或者层压制 品的胶原生物织物可以规则的间隔进行切割并将其张开，例如形成 网。

15 在一些实施方案中，胶原生物织物是平的，例如具有没有倾斜或 弯曲的表面。在其他实施方案中，本发明的胶原生物织物不是完全平 的，而是具有带有微凹的表面，例如在表面上有凹陷或凹入。

在一个特殊的、优选的实施方案中，本发明包括含有至少两层本 发明的胶原生物织物的层压制品。这些层可以物理方式结合在一起， 20 例如通过使用胶合剂、持着器，或者通过热压生物织物的一部分（例 如这些层中的至少一个的外周）以融合这些层。因为生物织物有"颗 粒"，所以生物织物可以多种方法层压成层压制品。在一个实施方案 中，所述层压制品的所有层具有同样的颗粒方向。在另一个实施方案 中，至少一个层的颗粒方向相对于至少另一个层的颗粒方向旋转了大 25 约90度（即大约垂直）。在一个更特殊的实施方案中，所述的垂直的层彼此相邻。在另一个实施方案中，所述的层压制品含有至少三层胶 原生物织物，其中所述至少三个层中的每一个的颗粒结构相对于余下 层中的两个的颗粒结构旋转了大约60度。在另一个实施方案中，所述 层压制品含有至少第一种材料和第二种材料，其中该第一种材料是本 发明的胶原生物织物，该第二种材料是任何其他能够与胶原生物织物 形成层压制品的物质。在一个特殊的实施方案中，所述第二种材料是 来源自除了羊膜之外的膜的片或膜。在另一个特殊的实施方案中，所 述第二种材料是非天然的材料，例如聚内酯、聚乙酸酯、塑料膜等等。

5 在一些实施方案中，羊膜层压制品是多层的，其含有至少6层、至少8 层、至少10层、至少20层、至少80层、至少100层、至少1000层 根据本发明的方法制备得到的羊膜。本发明的羊膜层压制品可以含有 无限数量的层。

pipes. In other embodiments, the present invention provides a biological collagen fabric of the present invention, the laminate further comprises a hydrogel composition or composition of their 30 three-dimensional scaffold.

Accordingly, the present invention provides a variety of forms and configurations biological collagen fabric, laminates comprising, scaffolds, and hydrogel composition, but is not limited thereto. The present invention provides any useful medically form of the composition of the present invention. Regardless of the particular form or structure, the composition of the present invention may further comprise one or more biological molecules, preferably a therapeutic agent. The composition being described in detail herein, the present invention containing biological molecules in the medical field has many uses. In some embodiments, the present invention comprises a composition of the present invention 5 transplantation of living cells, so these cells are uniform and confluent. The composition of the present invention is transplanted cells in the medical and dental field has many uses, such as for tissue engineering purposes.

The present invention further relates to the preparation of biological collagen fabric, a laminate thereof, which method scaffolds or hydrogel composition. In one particular embodiment, the present invention provides a placenta with 10 from amnion and chorion collagen prepared biological fabric, comprising: separating layer and chorionic amnion layer; and the decellularized amniotic membrane, amniotic membrane that does not enzymes such as proteases contacts.

In other embodiments, the method also requires washing and drying the cell membrane of the sheep go. In another particular embodiment, the present invention provides a method from the method of preparation of amniotic membrane laminate having a placenta amnion and chorion, comprising: separating layer and chorionic amnion layer; the decellularized amniotic membrane 15; and at least the decellularized amniotic membrane layers in contact with each other stacked together to form a laminate amnion. In a particular embodiment, the decellularized amniotic membrane is dried before stacking. In another particular embodiment, the decellularized amniotic membrane is dried after lamination, such that at

层压制品具有增加的结构刚性，这使得层压制品能够塑造成复杂 10 的三维结构。这种三维结构可以包括片、管、微球体。

胶原生物织物还可以单片或者层压制品的形式与另一种材料联合 在一起。例如，生物织物可以与可屈曲的塑料膜、纱布、塑料片、支 架、瓣膜、矫形装置、绷带、贴剂等等联合即结合在一起。

股原生物织物可以含有一种或多种不为生物织物的胶原基质的部 15 分的化合物或物质。例如，胶原生物织物可以在生产或者外科程序准 备期间用生物分子进行浸渍。这种生物分子包括抗生素（例如克林霉 素、二甲胺四环素、强力霉素、庆大霉素）、激素、生长因子、抗肿 瘤剂、抗真菌剂、抗病毒剂、疼痛药物、抗组胺剂、抗炎剂、抗感染 剂（包括银（例如银盐包括硝酸银和磺腰嘧啶银，但并不局限于此）、 20 元素银、抗生素、杀菌的酶（例如溶菌酶），但并不局限于此）、伤 口愈合剂（例如细胞因子包括PDGF、TGF,但并不局限于此；胸腺素）、 作为伤口愈合剂的透明质酸、伤口封闭剂（例如有或没有凝血酶的血 纤蛋白）、细胞吸引剂和支架试剂（例如纤连蛋白）等等，但并不局 限于此。在一个特殊的实施方案中，胶原生物织物可以用至少一种生 25 长因子进行浸渍，例如成纤维细胞生长因子、上皮细胞生长因子等等。

生物织物还可以用小有机分子进行浸渍，例如特殊生物化学过程的特 异性抑制剂，如膜受体抑制剂、激酶抑制剂、生长抑制剂、抗癌药物、 抗生素等等。

在另一个实施方案中，本发明的股原生物织物可以联合水凝胶。

30 本领域熟练技术人员已知的任何水凝胶组合物包括在本发明的之内， 例如在下列综述中所揭示的任何水凝胶组合物：Graham，1998，Med. Device Technol. 9(1): 18-22; Peppas 等人，2000，Eur. J. Pharm. Biopharm. 50(1)： 27-46； Nguyen 等人，2002，Biomaterials, 23 (22): 4307-14;Henincl 等人，2002, Adv. DrugDeliv. Rev 54 (1)： 13-36; Skelhorne 等人，2002, Med. Device. Technol. 13(9): 19-23; Schmedlen 等人，2002' Biomaterials 23: 4325-32;所有 5 这些文献在此处以其整体进行引用作为参考。在一个特殊的实施方案 中，水凝胶组合物应用于胶原生物织物之上，即放置于胶原生物织物 的表面上。水凝胶组合物例如可以喷洒在胶原生物织物上、布满生物 织物的表面、浸泡胶原生物织物、浸洗胶原生物织物、或者包被至胶 原生物织物的表面。

least two decellularized amniotic membrane contact with each other.

20 The present invention provides methods of using the present invention in medical, dental and surgical environment collagen bio-textiles, laminated products, its method of three-dimensional scaffold or hydrogel composition. In fact, the desired composition of the present invention is known in the art with respect to other biological materials having enhanced treatment and clinical utility. In some embodiments, the present invention provides a method of the present invention, compositions for the treatment and / or prevention of a disease or disorder related eye in a subject. In a particular embodiment 25, the present invention provides a method of treating in a subject and / or prevention of eye-related disease or disorder, comprising the biological fabric placed on the surface of the eye disease subjects.

In other embodiments, the present invention provides a method of treatment and / composition of the present invention in a subject (preferably human) or prevention of skin conditions. In one particular embodiment, the method comprises contacting the composition with the subject's skin. In another particular aspect of the embodiment 30, the composition is placed directly on the skin surface of the subject, the skin surface is a portion of the skin condition.

The present invention also provides a method of treating a wound or burn in a subject (preferably a human) in, which includes the site of the wound or burn in contact composition.

In other embodiments, the present invention provides methods of using the present invention is a composition for use in surgical procedures (such as collagen bio-textiles, amniotic membrane laminate), for example in eye surgery, cardiovascular surgery, periodontal surgery, neurosurgery, dental surgery and orthopedic surgery.

5 The present invention provides methods of using the compositions of the present invention to deliver biomolecules (preferably a therapeutic agent) to a subject (preferably a human) method. The present invention also includes the use of the compositions of the present invention is a method for delivering a cell to a subject (preferably a

10 在本发明的方法和组合物中可用的水凝胶可以从任何本领域中已 知的与水相互作用或者水溶性的聚合物制得，包括聚乙烯醇（PVA)、 聚羟乙基异丁烯酸酯、聚乙二醇、聚乙烯吡咯烷酮、透明质酸、葡聚 糖或者其衍生物和类似物，但并不局限于此。

在一些实施方案中，本发明的胶原生物织物进一步在与水凝胶联 15 合之前用一种或多种生物分子进行浸渍。在其他实施方案中，水凝胶 组合物进一步在与本发明的胶原生物织物联合之前用一种或多种生物 分子进行浸渍。这种生物分子包括抗生素（例如克林霉素、二甲胺四 环素、强力霉素、庆大霉素）、激素、生长因子、抗肿瘤剂、抗真菌 剂、抗病毒剂、疼痛药物、抗组胺剂、抗炎剂、抗感染剂（包括银（例 20 如银盐包括硝酸银和磺胺嘧啶银，但并不局限于此）、元素银、抗生 素、杀菌的酶（例如溶菌酶），但并不局限于此）、伤口愈合剂（例 如细胞因子包括PDGF、TGF,但并不局限于此；胸腺素）、作为伤口 愈合剂的透明质酸、伤口封闭剂（例如有或没有凝血酶的血纤蛋白）、 细胞吸引剂和支架试剂（例如纤连蛋白）等等，但并不局限于此。在 25 一个特殊的实施方案中，胶原生物织物或者水凝胶组合物可以用至少 一种生长因子进行浸渍，例如成纤维细胞生长因子、上皮细胞生长因 子等等。优选地，生物分子为治疗剂。

在一些实施方案中，水凝胶组合物与含有本发明的生物织物的层 压制品联合。

30 水凝胶/胶原生物织物组合物在医学领域中具有用途，包括治疗创 伤、烧伤和处理皮肤状况（例如处理瘢痕），以及用于美容用途（例 如美容外科）和作为植入物的任何用途，但并不局限于此。在一些实 施方案中，水凝胶/胶原生物织物组合物局部施用于受试者，即施用在 皮肤表面上，以例如用于治疗创伤。在其他实施方案中，水凝胶/胶原 生物织物组合物可以在受试者的内部使用，例如作为植入物，以便成 为体内永久性或半永久性的结构。在一些实施方案中，将水凝胶组合 5 物配制成不可生物降解的。在其他实施方案中，将水凝胶组合物配制 成可生物降解的。在一个特殊的实施方案中，将水凝胶组合物配制成 可在几天之内降解。在另一个特殊的实施方案中，将水凝胶组合物配 制成可在几个月之内降解。

在一些实施方案中，在本发明的胶原生物织物上移植细胞，以致 10 于这些细胞是均一的和汇合的。可以用于在本发明的生物织物上移植 的细胞包括干细胞（优选为人干细胞）、人分化的成熟细胞、全能干 细胞、多能干细胞、多潜能干细胞、组织特异性干细胞、胚胎样干细 胞、定向祖细胞、类成纤维细胞，但并不局限于此。在其他实施方案 中，本发

human), wherein the composition has further been transplanted cells.

5. 1 share protozoa fabric 10 of the present invention provides a collagen amnion, herein referred to as biological collagen fabric. Collagen biological fabric of the present invention to maintain a natural, untreated amniotic structural integrity, that is to maintain the structure of proteins (such as collagen, elastin, fibronectin perhaps) the tertiary and quaternary structure in its composition. Accordingly, the present invention is collagen bio-textiles made of natural or untreated amniotic membrane like structure composed of proteins. The prior art method requires the use of a protease produced or amniotic 15 hyperthermic treatment, so these membranes can not be maintained and the tertiary structure of the four-level structural protein in their composition.

In one particular embodiment, the present invention provides a dehydrated, decellularized and no matrix (ie no filter back village) amniotic membrane, amniotic tertiary and quaternary structure therefore has a natural (see Figure 2A and B). Dehydration of the present invention, the decellularized amniotic membrane is uniform (ie a minimum of 20 to cellular material), translucent fabric creature, which has the appearance shown in Figure 3, which has a decellularized matrix left-handed triple helix a helical segments (see e.g., Molecular Biology of the Cell, 1989, Alberts et al (editors), Garland Publishing company, New York, NY; herein incorporated by reference in its entirety).

25 ? collagen bio-textiles invention can source from any mammalian amniotic membrane, such as horses, cattle, pigs or narrow nose animal sources, but the most preferred source is from human placenta. Natural tertiary and quaternary structure in a preferred embodiment, the present invention has a biological fabric collagen material in its composition. are different from those described in the prior art, the biological collagen fabric of the invention to operate with a minimum limit 30, i.e. the collagen fabric of the present invention is bio-up of a chemical or biological treatments or operations, e.g., in a weak detergent performed decellularization. When used herein, the "minimum operation" refers to any step during the preparation of biological collagen fabric, the present invention does not amnion processing enzyme such as a protease treatment, high heat

明包括在本发明的生物织物上移植特殊种类的祖细胞，包括 15 软骨细胞、肝细胞、造血细胞、胰实质细胞、成神经细胞和肌肉祖细 胞，但并不局限于此。

5.2生产胶原生物织物的方法 本发明提供了制备本发明的胶原生物织物的方法。特别地，本发 明包括制备胶原生物织物的方法，其包括：提供含有羊膜和绒毛膜的 20 胎盘；分离羊膜和绒毛膜；和将羊膜进行去细胞化。在一个特殊的实 施方案中，该方法此外还需要洗涤和干燥经去细胞化的羊膜。

优选地，胎盘来自人胎盘以在人受试者中使用。在一些实施方案 中，本发明包括来自动物物种的胎盘以在人受试者中使用。在其他实 施方案中，本发明包括来自动物物种的胎盘以在动物受试者中进行兽 25 医学应用。

在优选的实施方案中，在本发明的方法中使用的胎盘在新生儿分 娩之后尽可能快地取得。在另一个优选的实施方案中，胎盘在剖腹产 术分娩出正常健康的婴儿之后紧接着取得胎盘。如果在无菌的条件下收集胎盘。在一些实施方案中，胎盘在任何进一步的处理之前从分娩 30 的时候起贮存48小时。在其他实施方案中，胎盘在任何进一步的处理 之前从分娩的时候起贮存直至5天。

优选地，将胎盘、脐带和脐带血从分娩或生育室转移至另一个地 点例如实验室以便进一步的处理。胎盘优选地转移至无菌的运输装置 中，例如无菌的袋或容器中，其可选地是隔热的。在一些实施方案 中， 将胎盘于室温贮存直至进一步的处理。在其他实施方案中，将胎盘进 行冷藏直至进一步的处理，即于大约2-8X：的温度贮存。在其他实施方 5 案中，将胎盘于进一步处理之前在无菌条件下贮存直至5天。在一个 最优选的实施方案中，正如本领域熟练技术人员已知的，在无菌条件 下操作和处理胎盘。实验室优选地装备HEPA过滤系统（如洁净室分级 所定义的，具有1000级或更好）。在一个优选的实施方案中，在使用 实验室之前将HEPA过滤系统开启至少1小时以便施行本发明的方法。

10 在某些实施方案中，去除胎盘中的血，即完全排干生育之后剩余 的脐带血。在一些实施方案中，胎盘中血的去除率为70%、80%、90%、 95%、99%。

本发明包括在生育之前用本领域熟练技术人员已知的标准技术来 对孕妇进行筛选以检查传染病，包括HIV、HBV、HCV、HTLV、梅毒、 15 CMV和其他已知会污染胎盘组织的病毒病原体，但并不局限于此。优选 地，用于筛选传染病的方法遵循联邦药品

treatment, strong chemical treatment, exposure to decontamination agent or acid. As part of the steps to the cells, amniotic membrane protein enzyme treatment can damage the structural integrity of the biological fabric, such as the impact tertiary and / or quaternary structure of the collagen material. In contrast, minimum 5 operating in a biological amniotic membrane fabric preparation of the present invention result obtained with respect to the prior art products, products with enhanced mechanical strength and translucent products. decellularized matrix of the present invention and non-biological collagen fabrics contain collagen (type I, IV and type II collagen, but not limited to) as well as fibronectin and elastin.

This is in part caused by a joint in accordance with the method described herein, the present invention is obtained by preparing biological tissue 10 having increased mechanical strength reasons. As a result of the operation of the processing and minimal collagen biological fabric of the present invention maintains the natural composition of the film. Collagen is the main structural material of vertebrate, which has a major presence in the mechanical function of the organization. Collagen molecule consists of three polypeptide chains known as a chain (each of about 1,000 amino acids in length) which, in the three chains as triple strand intertwined with each other in the same, thereby forming the left-hand rule supercoiled. 15 already identified at least 19 types of collagen, they all have the same three-dimensional fabric (see, for example, Lee et al., 2001, International J. of Pharmaceutics, 221: 1-22) "of the present invention in the form of amniotic membrane, biological mechanical and structural characteristics as reported in the prior art known to the inventors and those who want more than good, so this part is based on the discovery of a novel method at 20 Preparation of human placenta and amniotic controlled and adequate source (see Section 5. 2).

In particular, as compared to one or more of the amnion prior art, the biological collagen fabric of the invention having one or more of the following characteristics: tensile strength enhancement; seamable excellent properties; reduced immunogenic, which results in a decrease of host - graft rejection; easily stored and transported, without freezing or cryopreservation; after activation and operation require minimal

管理局所提出的规章。孕妇 可以在生育的一个月之内进行筛选（即取得血液样品用于诊断目的），优选地在生育的两个星期之内，甚至更优选地在生育的一个星期之 内，和最优选地在生育的时候。只使用从这样的供体中收集得到的组 20 织来生产本发明的生物织物，即其母亲经检测对于上面提及的病原体 呈阴性或者无反应性。优选地，获得胎盘膜的供体的详细的父亲以及 医疗和社会历史资料，包括例如详细的家族史。

使用本领域熟练技术人员已知的标准血清学和细菌学测试来筛选 供体。任何鉴定病原体的测定或诊断测试在本发明的方法的范围之 25内，但是优选的测定为那些联合了高精确度与高处理量能力的测定。

在一个特殊的实施方案中，本发明包括用本领域熟练技术人员已知的 标准技术来对供体进行歸选以检查抗原和/或抗体。抗原和抗体的非限 制性例子包括：抗体筛选（ATY)；丙氨酸转氨酶筛选（ALT)；肝炎 核心抗体（核酸和ELISA)；乙型肝炎表面抗原；丙型肝炎病毒抗体； 30 HIV-I 和 HIV-2; HTLV-I 和 HTLV-2;梅毒测试（RPR)； CMV 抗体测试；以及丙型肝炎和HIV测试。所使用的测定可以是基于核酸的测定或者 基于ELISA的测定，这些对于本领域熟练技术人员来说是已知的。

本发明此外还包括用本领域熟练技术人员已知的标准技术来检测 来自新生儿脐带的血液（见例如，Cotorruelo等人，2002, ClinLab. 48 (5-6): 271-81; Maine 等人，2001, Expert Rev. Mol. Diagn., 1(1): 19-29; Nielsen 等人，1987, J. Clin. Microbiol. 25(8): 5 1406-10;所有这些文献在此处以其整体进行引用作为参考）。在一个 实施方案中，用本领域熟练技术人员已知的标准技术来检测来自新生 儿脐带的血液以检查细菌病原体（包括革兰氏阳性和革兰氏阴性细 菌，但并不局限于此）和真菌。在一个特殊的实施方案中，用本领域 熟练技术人员已知的标准技术来测定新生儿脐带血的血型和Rh因子。

10 在另一个实施方案中，用本领域熟练技术人员已知的标准方法从来自 新生儿脐带的血液中获得具有差异的CBC。在另一个实施方案中，用本 领域熟练技术人员已知的标准方法从来自新生儿脐带的血液中取得需 氧细菌培养物。只使用从这样的供体中收集得到的组织来生产本发明 的生物织物，即其具有在正常限度内的CBC (例如没有显著的离开正常 15 水平的异常或偏差），经检测在血清学和细菌学上是阴性的，和经检 测对于传染病和污染呈阴性或无反应性。

用于制备本发明的胶原生物织物的范例性方法包括下列步骤。

preparation, i.e. 25 rehydration prior to use program; and it can be stored at room temperature for an extended period of time, while maintaining the integrity of the structure and function. Table 1 summarizes the advantages of the biological fabric of the present invention and the prior art when compared to those obtained.

In an alternative embodiment, the present invention provides a composition containing dehydrated, decellularized matrix and no chorionic (preferably human chorionic) collagen bio-fabric. Expect collagen-containing biological membrane fabric cashmere wool 30 will have a fabric of the present invention contain collagen biological amnion quite characteristic. The present invention provides homes have chorionic collagen bio-textiles all medically useful forms, including laminates, scaffolds, and hydrogel composition, but is not limited thereto. amnion collagen bio-textiles traditional serological tests Table 1. biological fabric of the present invention and the conventional system amniotic 3 Comparative characteristics of the present invention fully Journal: Antibody screening (ATY); alanine aminotransferase Screening (ALT); hepatitis core antibody (nucleic acids and ELISA); hepatitis B surface antigen (nucleic acids and ELISA); hepatitis C virus antibody (nucleic acids and ELISA); HIV-I and HIV-2; HTLV-I and HTLV-2; syphilis test (RPR); CMV antibody test; hepatitis C and HIV testing (nucleic acid test) incomplete: no CMV test to save drying: dehydration with low heat and vacuum; without freezing or refrigeration equipment; freeze-frame storage: in a medium storage; transportation needs of dry ice and freezer / refrigeration equipment surgery a few minutes to prepare: ready within a few minutes; the surgical site directly in Sheung Shui; for example hydrated salt water drops in the eye; without melting, soaking or rinsing long time: requires a lot of preparation / thaw / soaking time; filter must be removed simply backing surgical operation: can be trimmed while drying; and applied to the surgical site, and then at the site of heavy Sheung Shui: You must remove the filter paper; during the surgical procedure tend to "group into a ball"; difficult to perform surgical operations on cell decellularized: not least to epithelial cells; minimal to no cell debris; rapid reconstruction contains dead cells: the presence of dead cells; clinical evidence that a longer healing time / high rejection rate (anecdotal evidence) matrix None: None matrix (for example,

步骤I。本发明包括处理胎盘膜，以便（可选择地）从胎盘中分离 出脐带，和分离羊膜和绒毛膜。在一个优选的实施方案中，在切割胎 20 盘膜之前将羊膜和绒毛膜进行分离。羊膜和绒毛膜的分离优选地从胎 盘膜的边缘开始进行。在另一个优选的实施方案中，使用钝器解剖来 分离羊膜和绒毛膜，例如用戴着手套的手指。在将羊膜与绒毛膜和胎 盘分离之后，例如用剪刀切割下脐带残端，并从胎盘上移开。在某些 实施方案中，当不撕裂组织就不可能分离羊膜和绒毛膜时，本发明包 25 括将羊膜和绒毛膜作为一块从胎盘上切割下来，然后将它们剥离开。

然后优选地将羊膜贮存于无菌盐溶液中。在一些实施方案中，无 菌盐溶液是缓冲溶液。在一个特殊的优选实施方案中，用于贮存羊膜 的无菌盐溶液为0. 9%无菌NaCl溶液。优选地，通过于至少41C的温度 进行冷藏来贮存羊膜。在某些实施方案中，羊膜于至少21C、至少61C 30 或者直至8TC的温度进行冷藏。在这一点上，如果将羊膜冷藏并保持覆 盖有无菌盐水，那么羊膜可以贮存直至5天。优选地，在程序中的下 一个步骤之前将分离的羊膜从分娩的时候起冷藏至多72小时。

步骤II。一旦将羊膜与绒毛膜分离，本发明就包括将羊膜进行去 细胞化。本发明的方法包括任何本领域熟练技术人员已知的去细胞化 方法，但规定用于将本发明的羊膜进行去细胞化的方法不包括任何冷冻羊膜的步骤。在此处使用时，去细胞化是指从本发明的羊膜上去除 5 所有细胞材料和细胞碎片（例如所有可见的细胞材料和细胞碎片）。

羊膜的去细胞化确保基本上所有通常与羊膜的胶原基质相联系的细胞 都被去除。本发明的羊膜的去细胞化去除了“基本上所有”与胶原基 质相联系的细胞，其优选地去除至少90%的细胞，更优选地去除至少 95%的细胞，和最优选地去除至少99%的细胞。根据本发明的方法进行 10 去细胞化的羊膜是均一地薄的即厚度为10-40微米，是光滑的（通过触摸测定）和在外观上是清澈的。

在一个优选的实施方案中，本发明的羊膜的去细胞化包括从羊膜 的母体侧去除基本上所有细胞材料和细胞碎片，随后从羊膜的胎儿侧 去除所有细胞材料和细胞碎片。在一个特殊的实施方案中，本发明的 15 羊膜的去细胞化包括物理性刮削，并联合使用无菌溶液的漂洗。在另 一个特殊的实施方案中，羊膜的物理性刮削包括使用无菌的细胞刮刀 进行刮削。在另一个特殊的实施方案中，用于在去细胞化期间漂洗羊 膜的无菌溶液为水溶液，即含有生理缓冲液或盐溶液的溶液，例如0. 9% NaCl溶液。

no filter backing) are: supported nitrocellulose / filter on the sterilization (optional) are: electron beam radiation; at least 18 kGy; increased organizational security to ensure no: store in a medium containing antibiotics and glycerol (toxic) in; no formal final sterilization of tissue transparency translucent: optically transparent opaque: milky or cloudy appearance in a preferred embodiment, the present invention collagen bio-fabric is translucent, that is optically transparent. In another preferred embodiment, the collagen biological fabric of the present invention is thin and light. In a particular embodiment, the dehydrated collagen raw material of the present invention 5 fabric is 0.3-0.6 mg / cm2. In a particular embodiment, the thickness of the collagen biological fabric of the present invention is at least 30 microns. In another particular embodiment, the thickness of the fabric of the present invention is the biological collagen is about 10-40 microns.

The present invention involves the use of a variety of configurations of biological collagen fabric of the present invention, such as inserts, guards. In general, given the biological fabric may be formed on any useful medical form.

10 For example, collagen bio-fabric can be set to use its entirety, using collagen bio-fabric from complete placenta. Alternatively, collagen bio-fabric can be cut into strips, sheets or rolls, or may be woven into line. In another embodiment, the thickness of a single sheet or a laminate of a biological collagen fabric may be cut at regular intervals and opened, e.g., forming a web.

15 In some embodiments, the collagen bio-fabric is flat, for example, has no inclination or curved surfaces. In other embodiments, the collagen biological fabric of the present invention is not completely flat, but with a slightly concave surface having, for example, on the surface of a depression or recess.

In a special, preferred embodiment, the present invention includes at least two layers of the present invention, the biological collagen fabric laminate. These layers can be physically joined together, e.g., by use of adhesives 20, holding unit, hot pressing or by biological fabric part (e.g. at least one of the layers of the outer periphery) of these layers to fusion. Since biological fabric of "particles", it may be a variety of biological methods laminated fabric laminate. In one

20 羊膜的去细胞化包括去除天然存在的细胞以及其他来自羊膜的抗 原和细胞碎片，和可选择地进行处理以抑制新的免疫位点的产生。在 对于羊膜进行去细胞化的过程中，去除天然的活细胞以及其他可能引 起不利的免疫反应的细胞和非细胞结构或成分。根据本发明所使用的 去细胞化技术不应当导致羊膜的解剖学的显著破坏，或者改变其结构 25 组分的生物机械特性，即胶原、弹性蛋白和还可能的纤连蛋白的结构 和生物化学完整性不受去细胞化的影响。明确地说就是，羊膜的强烈 化学处理和蛋白酶处理不在本发明的去细胞化技术的范围之内。

优选地，羊膜的去细胞化包括使用含有去污剂的溶液。根据本发 明的方法可以使用的去污剂包括非离子去污剂、Triton X-100、阴离 30 子去污剂或十二烷基硫酸钠，但并不局限于此。在本发明的方法中， 去污剂可以单独或联合使用。根据本发明的方法可以使用任何温和的 阴离子去污剂，即非腐蚀性的去污剂，其具有6-8的PH和低起泡性。

在一个特殊的实施方案中，在羊膜的去细胞化中使用0.01-1%的一水 合脱氧胆酸钠。虽然不想被特殊的作用模式所束缚，但是根据本发明 的方法，羊膜的去细胞化可能裂解了细胞膜，和有助于从羊膜上去除 细胞碎片。可是，应当采取措施来消除羊膜中的任何残留去污剂水平， 5 以便例如避免干扰后来在羊膜上再次移植活细胞。

根本的是在制备本发明的生物织物中限制蛋白酶的活性。添加剂 如金属离子螯合剂可形成对于许多蛋白酶来说不利的环境，例如1, 10-菲咯啉和乙二胺四乙酸（EDTA)。提供对于蛋白酶如胶原酶来说不最 适宜的条件可以有助于在裂解步骤期间保护羊膜组分如胶原不被降 10 解。对于蛋白酶来说不最适宜的条件可以通过这样来获得，即配制低 渗裂解溶液以去除或限制溶液中可用的钙和锌离子的数量。许多蛋白 酶在存在钙和锌离子的情况下是有活性的，而在没有钙和锌离子的环 境中失去许多它们的活性。优选地，低渗裂解溶液会通过选择下列条 件来制备，即pH、减少钙和锌离子的可用性、存在金属离子螯合剂和 15 使用对于胶原酶特异的蛋白酶抑制剂，从而该溶液将会最佳地裂解天 然存在的细胞，而同时保护下面的羊膜不会不利地被蛋白酶降解。例 如，低渗裂解溶液可能包括水的缓冲溶液，其PH为5. 5-8，优选为7-8, 无钙和锌离子，并含有金属离子螯合剂如EDTA。此外，在用低渗裂解 溶液处理羊膜期间，温度和时间参数的控制也可以用于限制蛋白酶的 20 活性。

优选的是，羊膜的去细胞化处理还限制了新免疫位点的形成。因 为胶原的酶降解据信导致增加的免疫原性，所以本发明包括用酶例如 核酸酶处理羊膜，

embodiment, all layers of the laminate have the same direction of the particles. In another embodiment, the at least one direction of the particle layer relative to the direction of the particle layer is at least one other large rotating 25 degrees to about 90 (i.e., approximately perpendicular). In a more specific embodiment, perpendicular to the layers adjacent to each other. In another embodiment, the laminate comprising at least three layers of biological collagen fabric structure wherein the particles of at least three layers with respect to each of the remaining two layers of the grain structure rotated approximately 60 degree. In another embodiment, the laminate comprises at least a first material and a second material, wherein the first material is a biological collagen fabric of the present invention, the second material is capable of any other biological collagen forming a laminate of fabric material. In a particular embodiment, the second material is in addition to the source from the amniotic membrane sheet or film. In another particular embodiment, the second material of said non-natural materials, such as polylactone, polyacetates, plastic film and the like.

5] In some embodiments, the amniotic membrane is a multilayer laminate comprising layers of at least 6, at least 8 layers, at least 10 layers, at least 20 layers, at least 80 layers, at least 100 layers, at least 1000 layers according to the present the method of the invention prepared amnion. Amniotic membrane laminate of the present invention can contain an unlimited number of layers. laminate having increased structural rigidity, which makes the laminate can be molded into complex three-dimensional structure 10. This three-dimensional structure may include sheets, tubes, microspheres.

Collagen biological or fabric may also form a monolithic laminate with another material joined together. For example, bio-fabric can be buckling with plastic film, gauze, plastic sheets, brackets, valves, orthopedic devices, bandages, patches, etc. That is the joint together. shares protozoa fabric may contain one or more non-biological portion of the fabric 15 points collagen matrix compound or substance. For example, collagen bio-fabric can be immersed in the production or preparation for surgical procedures during the use of biological molecules. Such biomolecules include antibiotics (such as clindamycin, minocycline, doxycycline, gentamicin), hormones, growth

这些酶在抑制细胞代谢、蛋白质产生和细胞分裂中是有效的，它们将羊膜组分的蛋白酶解降至最低，因此保存了羊膜的 25 下层结构。根据本发明的方法能够使用的核酸酶的例子为那些在消化 天然存在的细胞的DNA和RNA中有效的核酸酶，包括外切核酸酶和内 切核酸酶。根据本发明的方法能够使用的核酸酶的非限制性例子包括 抑制细胞活性的外切核酸酶如DNAase I (SIGMA Chemical Company, St. Louis, Mo.)和 RNAase A (SIGMA Chemical Company, St. Louis, 30 Mo.)，以及抑制细胞活性的内切核酸酶如EcoR I (SIGMA Chemical Company, St. Louis, Mo.)和 Hind III (SIGMA Chemical Company, St. Louis, Mo.)。更优选的是，将选择出的核酸酶在含有离子如镁、 钙的生理缓冲溶液中应用，这些离子对于核酸酶的活性是最适宜的。

优选地，缓冲溶液的离子农度、处理温度和处理时间长短由本领域熟 练技术人员通过常规的实验来选择，以确保所希望的核酸酶活性水 平。缓冲液优选地是低渗的，以促进核酸酶进入细胞内部。

5 在一个特殊的实施方案中，将本发明的羊膜进行去细胞化包括下 列步骤。首先，将羊膜转移至洁净的无菌容器中，并可选择地用无菌 水漂洗，然后用无菌纱布进行干燥。然后将羊膜置于无菌托盘上，并 且将母体侧朝上。用无菌的细胞刮刀（例如，32 cm, PE刀片，PS把 手，NalgeNunc International ）而通过从羊膜的母体侧物理性地去 10 除所有可见的细胞材料来将羊膜进行部分去细胞化。如果需要，使用 无菌水来帮助去除细胞和细胞碎片。在完成羊膜的母体侧的部分去细 胞化之后，将羊膜翻转，从而将胎儿侧朝上。用细胞刮刀轻轻地去除 胎儿侧上的所有可见的细胞碎片，在这过程中对于羊膜使用最小的压 力以防止撕裂。可以使用无菌水来帮助去除细胞和碎片。

15 在一个实施方案中，将去细胞化的羊膜在进一步处理之前置于无 菌容器中，该容器充满了无菌生理溶液例如无菌0.9% NaCl溶液。根 据本发明的方法，羊膜的下一步处理步骤应当不迟于将羊膜置于无菌 生理溶液中之后2-3小时开始施行。在一个特殊实施方案中，当步骤 III紧接着步骤II施行时，不需要将羊膜置于含有无菌溶液的容器 20 中。

在清洁过程期间，羊膜可以用例如无菌解剖刀进行切割，从而对 其进行形状修整以便更易清洁，或者去除不能清洁的区域。

步骤III。在去细胞化之后，洗涤羊膜以确保去除可能包括细胞蛋 白质、细胞脂类和细胞核酸在内的细胞碎片，以及去除任何细胞外碎 25 片如细胞外可

factors, anti-tumor agents, antifungal agents, antiviral agents, pain medications, anti-group histamines, anti-inflammatory agents, anti-infective agents (including silver (e.g., silver salts include silver nitrate and silver pyrimidinyl sulfonamide waist, but not limited to), 20 elemental silver, antibiotics, bactericidal enzymes (such as lysozyme), but is not limited to this), wound healing agents (e.g. cytokines include PDGF, TGF, but is not limited thereto; thymosin), hyaluronic acid as a wound healing agent, wound sealants (e.g., with or without thrombin fibrin), cellular attractant and bracket reagent (e.g. fibronectin) and the like, but is not limited thereto. In a particular embodiment, the collagen may be at least one biological raw fabric 25 impregnated with growth factors, e.g., fibroblast growth factor, epidermal growth factor, and so on.

Biological fabric may also be impregnated with small organic molecules, such as special-specific inhibitors of biochemical processes, such as a membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, and so on.

In another embodiment, the present invention shares protozoan fabric can be combined hydrogel.

30 known to a person skilled in the art hydrogel composition of any included within the present invention, e.g., any of the hydrogel compositions disclosed in the following review:. Graham, 1998, Med Device Technol. 9 (1): 18-22; Peppas et al., 2000, Eur J. Pharm Biopharm 50 (1):... 27-46; Nguyen et al., 2002, Biomaterials, 23 (22): 4307-14; Henincl et al., 2002, Adv DrugDeliv Rev 54 (1):.. 13-36; Skelhorne et al., 2002, Med Device Technol 13 (9):.. 19-23; Schmedlen et al., 2002 'Biomaterials 23: 4325 -32; 5 all of which are incorporated herein by reference in its entirety. In a particular embodiment, the hydrogel composition is applied on the biological collagen fabric, i.e., placed on the surface of the biological collagen fabric. E.g., the hydrogel composition can be sprayed on the biological collagen fabric, the fabric covered surface of the biological, bio fabric soaked collagen, collagen immersion biological fabric, or coated to the plastic surface of the fabric protista.

10 available in the methods and compositions of the present invention, the hydrogel may be known in the art from any interaction with water or a

溶性蛋白质、脂类和蛋白聚糖。虽然不想被任何作用机 制所束缚，但是去除这种细胞和细胞外碎片减少了羊膜的免疫原性。

一旦将本发明的羊膜进行了去细胞化，就进一步洗涤羊膜以便有 效地达到从羊膜的两侧完全地去除所有可见的细胞材料和细胞碎片。

该溶液优选为配制成可有效地裂解天然存在的组织细胞的低渗或低离 30 子强度水溶液。这种低渗水溶液可以为去离子水或者低渗水缓冲溶 液。此外，低渗水缓冲溶液优选地含有添加剂，这些添加剂提供了对 于蛋白酶的活性不最适宜的条件，例如可能由于细胞裂解而释放出的 胶原酶。

优选地，将羊膜在去污剂中轻轻地摇动，例如在摇动平台上，从 而帮助去细胞化。在某些实施方案中，将羊膜摇动至少15分钟、至少 20分钟、至少30分钟或者直至120分钟。在去污剂去细胞化之后，羊 5 膜可以再次如上所述进行物理性去细胞化；当需要时可以重复物理性 和去污剂去细胞化步骤，只要保持羊膜的完整性，直至没有剩下可见 的细胞材料和细胞碎片。

在一个特殊的实施方案中，羊膜的洗涤包括下列步骤：将去细胞 化的羊膜置于无菌容器中，然后用去细胞化溶液以足够覆盖羊膜的数 10 量充满容器；然后将含有羊膜和去细胞化溶液的容器置于摇动平台上 (例如 100 型，VWR Scientific Products Corp. , P.O. Box 640169, Pittsburgh, PA 15264-0169)。然后将去细胞化溶液中的羊膜在摇 动平台上摇动15-120分钟。在摇动步骤之后，将羊膜从容器中取出， 并置于充满无菌溶液如0.9% NaCl溶液的洁净的无菌托盘中。使用新 15 的无菌细胞刮刀来去除残留的去细胞化溶液，并从羊膜的两侧去除任 何剩余的细胞材料。该步骤可以按需要重复多次，以便从羊膜的两侧 去除所有可见的残留细胞材料。

在某些实施方案中，羊膜在去细胞化步骤之后紧接着（即在30分 钟内）进行干燥。可选择地，当不是立即进行进一步的处理时，羊膜 20 可以在干燥之前冷藏直至28天，例如贮存于2-8X：的温度。当去细胞 化的羊膜的贮存时间超过3天但少于28天时，覆盖羊膜的无菌溶液优 选地定时进行更换，例如每1-3天更换一次。

在某些实施方案中，当羊膜在洗涤之后未进行冷藏时，将羊膜在 开始施行制备的步骤IV之前洗涤至少3次。在其他实施方案中，当羊 25 膜已经进行了冷藏和无菌溶液已经更换过1次时，将羊膜在开始施行 制备的步骤IV之前洗涤至少2次。在其他实施方案中，当羊膜已经进 行了冷藏和无菌溶液已经更换

water-soluble polymer in the system, including polyvinyl alcohol (PVA), polyglycolic methacrylate, polyethylene glycol, polyvinyl pyrrolidone, hyaluronic acid, dextran or derivatives and analogs thereof, but is not limited thereto.

In some embodiments, the collagen biological fabric of the present invention is further associated with the hydrogel 15 before closing with one or more biological molecules impregnation. In other embodiments, the hydrogel composition before further joint with one or more biological molecules in biological collagen impregnated with the fabric of the present invention. Such biomolecules include antibiotics (such as clindamycin, two Yue amine tetracycline, doxycycline, gentamicin), hormones, growth factors, anti-tumor agents, antifungal agents, antiviral agents, pain medications, anti-group histamines, anti-inflammatory agents, anti-infective agents (including silver (e.g. example 20 and a silver salt comprising silver sulfadiazine, silver nitrate, but not limited to), elemental silver, antibiotics, bactericidal enzymes (such as lysozyme), but is not limited to this), wound healing agents (e.g. cytokines include PDGF, TGF, but is not limited thereto; thymosin), hyaluronic acid as a wound healing agent, wound sealants (e.g., with or without thrombin fibrin), cellular attractant and bracket reagent (e.g. fibronectin) and the like, but is not limited thereto. 25 In a particular embodiment, the bio-collagen hydrogel compositions or fabric can be impregnated with at least one growth factor, e.g., fibroblast growth factor, epithelial cell growth factor, and so on. Preferably, the biological molecule is a therapeutic agent.

In some embodiments, the hydrogel compositions of the present invention containing biological fabric laminated products jointly.

30 hydrogel / collagen bio-fabric composition has uses in the medical field, including the treatment of wounds, burns and treatment of the skin condition (such as processing scarring), as well as for cosmetic purposes (such as cosmetic surgery) and as an implant any of these purposes, but is not limited thereto. In some embodiments, the hydrogel / collagen fabric biological subject topically applied compositions, i.e., applied to the skin surface, for example for the treatment of wounds. In other embodiments, the hydrogel / collagen fabric biological subject compositions