



CHAPTER FOUR

New Insights into Vertebrate Skin Regeneration

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Abstract

Regeneration biology has experienced a renaissance as clinicians, scientists, and engineers have combined forces to drive the field of regenerative medicine. Studies investigating the mechanisms that regulate wound healing in adult mammals have led to a good understanding of the stereotypical processes that lead to scarring. Despite comparative studies of fetal wound healing in which no scar is produced, the fact remains that insights from this work have failed to produce therapies that can regenerate adult human skin. In this review, we analyze past and contemporary accounts of wound healing in a variety of vertebrates, namely, fish, amphibians, and mammals, in order to demonstrate how examples of skin regeneration in adult organisms can impact traditional wound-healing research. When considered together, these studies suggest that inflammation and reepithelialization are necessary events preceding both scarring

and regeneration. However, the extent to which these processes may direct one outcome over another is likely weaker than currently accepted. In contrast, the extent to which newly deposited extracellular matrix in the wound bed can be remodeled into new skin, and the intrinsic ability of new epidermis to regenerate appendages, appears to underlie the divergence between scar-free healing and the persistence of a scar. We discuss several ideas that may offer areas of overlap between researchers using these different model organisms and which may be of benefit to the ultimate goal of scar-free human wound healing.



1. INTRODUCTION

As a protective barrier from the outside world, the skin is injured more frequently than any other system. An explosion of interest into regenerative medicine has helped clinicians inch closer to the prospect of developing therapies to repair skin injuries, minor or severe. These include the transplantation of biofabricated skin, treatment with biological scaffolds, application of bioactive molecules and the introduction of stem cells with the aim of stimulating endogenous repair and regeneration mechanisms (Metcalf and Ferguson, 2007). Despite the rapid advances that regenerative medicine has achieved, the fact remains that humans cannot regenerate skin and the result of trauma, either minor or severe, is the formation of a scar (Bayat et al., 2003). Scarring can range from minor esthetic concerns to debilitating complications that severely compromise quality of life, and in the developed world alone, scarring occurs in 100 million patients annually (Bayat et al., 2003). The incidence of failure of wound healing in chronic wounds is rising sharply due to the aging population, diabetes, and obesity, and together these pose a major threat to public health and the economy of the United States (Sen et al., 2009).

In contrast to humans and most other mammals, tissue regeneration is frequently observed among animals in nature (Morgan, 1901). These animals have not only evolved mechanisms to staunch blood flow, seal off the wound following injury and thus survive an accident or a predator attack, but some have the ability to regenerate entire organs such as a limb. Clearly, the ability to regenerate complex organs must mean that these animals exhibit little to no scarring because scarring is a sign of regenerative failure. Therefore, we can learn a great deal about scar-free healing from these regenerative organisms, and indeed, interest in contemporary regenerative medicine has brought about a renaissance in the study of regeneration

biology. As regeneration biologists attempt to understand the mechanisms that regulate and drive organ regeneration in vertebrates, natural instances of skin regeneration are being explored as models for human skin regeneration and insights gained from these studies are the focus of this review.



2. BRIDGING REPAIR AND REGENERATION

Advancing the field of regenerative medicine, and scarless wound healing in particular, will ultimately depend on a deeper understanding of the cellular and molecular mechanisms that regulate natural regenerative phenomena in adults. Although these mechanisms are at present not well understood, a vast body of scientific literature focused on documenting instances of tissue regeneration across a broad phylogenetic spectrum may offer important clues as to cell types and molecular pathways that should be targeted toward developing new therapies to treat traumatic skin injuries and excessive fibrotic conditions. Similarly, insights from applied studies that use synthetic matrices, biological scaffolds and stem cells applications, are equally important for their clinical relevance toward inducing a regenerative response (Metcalf and Ferguson, 2007). When results from these approaches are integrated under one umbrella, a new regeneration paradigm emerges at the interface of these tangential fields.

As this constantly evolving research paradigm develops, it is vital that wound-healing researchers, regeneration biologists, and clinicians move forward with a common set of operational definitions that will allow comparison and interpretation of experimental findings across different animal and experimental models. In order to clarify these operational definitions, it is helpful to consider a continuous spectrum that explains the outcome of skin trauma. At one end of this spectrum is *scarring*, the usual outcome of skin injury in mammals, while at the other end lies *regeneration*. As defined in mammals, a scar occurs following almost every dermal injury where its production obliterates the organs that resided within it along with the structure of the original extracellular matrix (ECM; Bayat et al., 2003; Metcalf and Ferguson, 2007). Although new ECM is produced, it usually exhibits reduced tensile strength and the majority of collagen fibers are densely compacted, running parallel to the wound epidermis (Yannas, 2001). In addition, epidermally derived organs such as hair follicles and sweat glands do not reappear in a scar. The term scarring is often used interchangeably with fibrosis, where fibrosis refers to the *excessive production of connective tissue*. However, fibrosis can also refer to the positive production of new ECM in

the wound bed. As summarized later in this review, new ECM appears similarly during skin regeneration and normal wound healing, except during skin regeneration, this early fibrosis is followed by remodeling that does not result in a scar. For this reason, we use fibrosis to describe the production of fibrous tissue at any time during skin repair or regeneration with no negative connotation.

At the other end of the continuum, *regeneration* broadly refers to the functional replacement of injured tissue, although its broad usage can often lead to inappropriate comparisons without qualification. Regeneration is properly referred to as *physiological* (in the absence of injury) or *reparative* (in response to injury) (Morgan, 1901). Physiological regeneration refers to the regular and repeated replacement of cells and tissues in an organism during normal homeostatic maintenance and occurs in almost every animal on planet Earth (Hay, 1966; Morgan, 1901). Examples abound (Seifert et al., 2012b) and include the regular replacement of skin epithelium, hair follicles, the annual replacement of antlers, and the constant replenishment of blood cells.

In contrast to physiological regeneration, reparative regeneration occurs in response to injury or amputation and leads to the functional replacement of the missing structure. Based on comparative studies, reparative regeneration can be further subdivided into regeneration that occurs either without (morphallaxis) or with (epimorphosis) the involvement of cell proliferation (Alvarado, 2000; Morgan, 1901). Morphallaxis is found to occur rather infrequently, being typified by *Hydra* regeneration where local cells at the site of injury reorganize and repattern themselves to replace the missing tissue without the need for proliferation to supply the injured area with a source of new cells. In contrast, all known examples of vertebrate regeneration involve cell proliferation. Furthermore, epimorphic regeneration can occur with or without formation of a blastema, a lineage-restricted mass of progenitor cells that give rise to the missing structure (reviewed in Monaghan and Maden, 2013). The archetypal example of blastema formation is that of the regenerating amphibian limb and a blastema forms during appendage regeneration in many metazoan taxa (Goss, 1969). Importantly, the formation of a blastema sets the stage for a series of events that appear to recapitulate the embryonic development of the missing structure.

In contrast to blastema-based regeneration, other examples of vertebrate regeneration (e.g., lens, heart, liver, skin) appear to require cell proliferation, but progress in an organ-specific manner. For example, regeneration of a lens proceeds by proliferation of pigmented retinal epithelial cells of the dorsal iris which then differentiate into a new lens (Eguchi and Shingai, 1971).

Heart regeneration in zebrafish occurs through localized fibrosis to patch the injured ventricle, followed by proliferation and migration of cardiomyocytes to the site of injury and finally removal of the provisional matrix and new muscle formation to produce a functional replacement of the injured tissue (Gonzalez-Rosa et al., 2011; Poss et al., 2002). What makes the nuances between these definitions so important in the context of skin regeneration is that they support the notion that the regenerative process of one organ does not necessarily imitate the regenerative process in a different organ. Thus, while there are surely genetic commonalities between limb and skin regeneration, especially at early inflammatory stages (Monaghan et al., 2012), routinely drawn comparisons between blastema-dependent limb regeneration and scar-free healing in mammals are likely to lead to conceptual problems that impede, rather than enhance, translation of these ideas to regenerative medicine. Therefore, as will be outlined below based on comparative data, we propose that vertebrate skin regeneration is a blastema-independent form of epimorphic regeneration.

Properly defining the process of skin regeneration among the different modes of regeneration can help make sense of how skin wounds heal in the myriad types of wound models used in mammalian wound-healing experiments. For instance, physiological regeneration of the skin epithelium occurs continuously throughout life, and thus it comes as no surprise that reepithelialization (i.e., epidermis regeneration) occurs in all wound types other than pathological conditions such as nonhealing wounds or diabetic ulcers. Similarly, hair follicles are replaced via physiological regeneration, and thus partial thickness wounds that remove only part of the dermis, leaving behind the base of hair follicles, also regenerate quite well (Yannas, 2001). However, in most mammal studies, full-thickness excisional wounds where epidermis, dermis, and hypodermis are removed do not regenerate and heal with scarring. Pertinent to this review is whether these wounds have a natural capability to regenerate that is suppressed and if so, can the repair process be coaxed along the continuum from scarring toward regeneration? In order to gain this insight, we can look to a diverse array of new and old vertebrate models of wound healing to see how the natural process of skin regeneration occurs in adults.



3. OVERVIEW OF MAMMALIAN WOUND REPAIR

For some time, we have enjoyed a descriptive understanding of the cellular events that occur during mammalian skin repair (Clark, 1996).

Building and expanding on this work, we have developed a more thorough understanding of the molecular control of these processes and are beginning to develop a detailed picture of the dynamic interaction of cells and their environment in the wound bed (Schultz et al., 2011). Nevertheless, the result of full-thickness injuries to mammalian skin is the formation of a dermal scar that completely ablates the original tissue architecture (Fig. 4.1). Understanding how and why a scar forms is the basis for potential insights into how regeneration might be achieved, especially when considered in the context of wound repair in animals that are capable of regenerating skin (Seifert et al., 2012c). For a complete and in-depth review of mammalian skin repair, we direct the reader to Clark's book on the topic (Clark, 1996) and several more contemporary reviews (Schultz et al., 2011; Werner and Grose, 2003). Below, we briefly review some of the major processes involved and then turn our attention to skin healing and regeneration in a number of nonhuman vertebrates.

3.1. Hemostasis and inflammation

Following the blood vessel disruption that necessarily follows skin wounding, platelets flow into the wound site, aggregate, degranulate, and coagulate to form a platelet plug or thrombus (Rivera et al., 2009). One of the final steps in the coagulation cascade is the conversion of blood-derived fibrinogen to fibrin via thrombin (Clark, 2003). Accumulation of fibrin produces a fibrin clot that will act as a provisional matrix for inflammatory cells and provide a substrate for epithelial migration (Fig. 4.1). In addition, it will become a reservoir for a multitude of growth factors and chemokines including bFGF, PDGF, VEGF, TGF β , and heparin, which it binds to either directly or through its association with ECM components. These factors allow the fibrin clot to continue to provide a matrix for the recruitment of cells to the injured site. In this way, inflammatory leukocytes, neutrophils, and monocytes (which become macrophages) migrate and enter the wound site.

Neutrophils act as early inflammatory sentries, responding to proinflammatory cytokines, chemical cues, and bacterial fragments at the injury site. These cells destroy bacteria by phagocytosis, and if substantial contamination has not occurred, neutrophil invasion will cease within a few days. Monocytes begin to infiltrate the wound behind neutrophils, and monocyte accumulation is stimulated by many chemoattractants including fragments of collagen, elastin, fibronectin, thrombin, and several growth

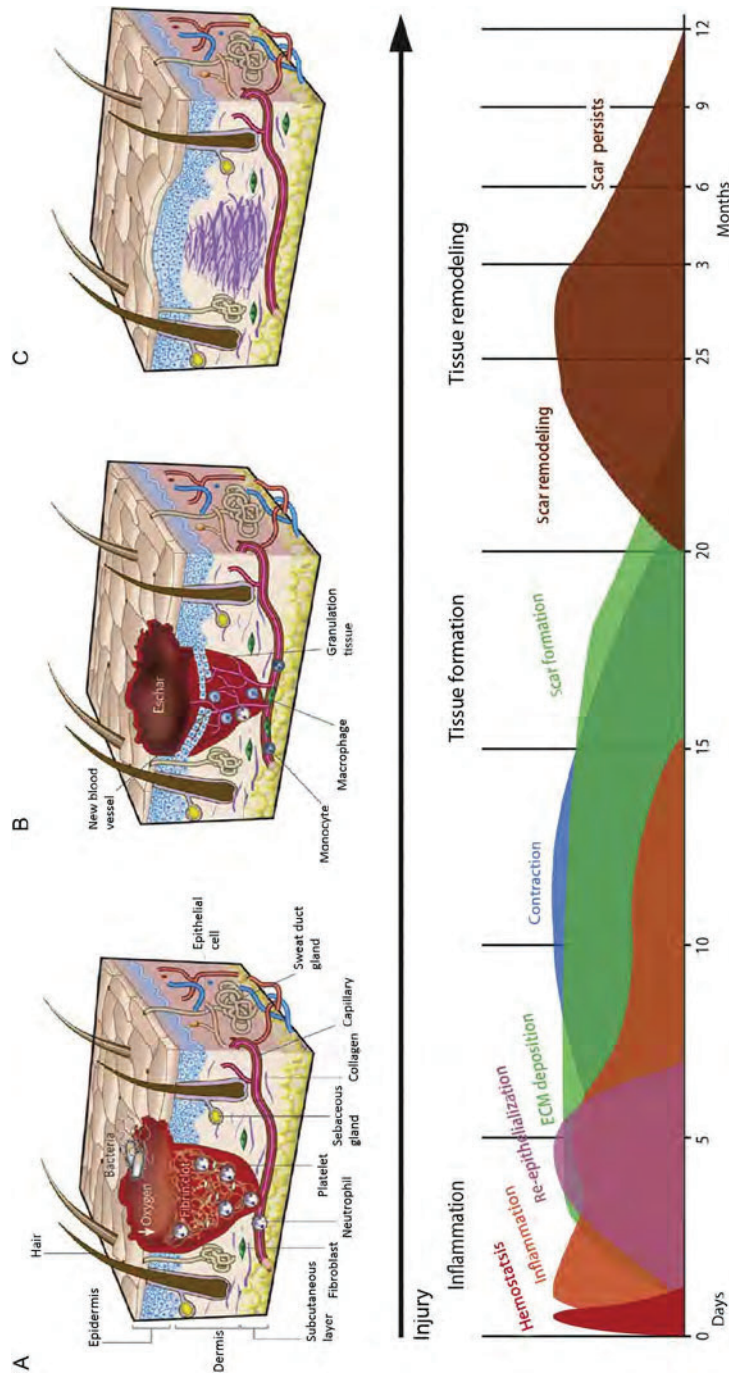


Figure 4.1 Wound healing of 4-mm full-thickness excisional wounds in mammals. (A) Hemostasis and inflammation, 0–48 h after injury. Immediately after wounding during inflammation, a fibrin clot forms that contains bacteria, degranulated platelets, inflammatory cells, and many growth factors. (B) Reepithelialization, 2–6 days after injury, and new tissue formation, 2–21 days after injury. A scab (eschar) has formed on the surface under which basal keratinocytes from the wound margins migrate to reepithelialize the wound. New blood vessels infiltrate the fibrous granulation tissue of the wound site. (C) Tissue remodeling, up to 1 year. A region of disorganized collagen, mostly type 1 collagen, is laid down by fibroblasts. This dense network of collagen will form the scar, which is often raised relative to the surrounding surface. The original architecture of the skin is completely ablated by the scar; there are no hairs, sweat glands, or sebaceous glands present in the repaired region. Colored peaks at the bottom represent the range of time periods of individual events during wound healing from multiple studies. The Y-axis represents percent maximal response for indicated process. Adapted from [Gurtner et al. \(2008\)](#).

factors such as TGF β and PDGF (Riches, 1996). As monocytes enter the wound bed, they mature into macrophages and phagocytose pathogens, tissue debris, and spent neutrophils. In addition, macrophages release potent ECM-degrading enzymes such as collagenase and other proteinases that help facilitate their movement throughout the fibrin clot and provisional matrix (Riches, 1996). Macrophages are a source of many growth factors, including TNF- α , PDGF, TGF- β 1, IL-1, TGF- α , IGF-1, and FGFs, which stimulate proliferation of fibroblasts, myofibroblasts, and endothelial cells (Barrientos et al., 2008). Some of these factors are necessary for new tissue formation and repair, and so this period marks the transition from inflammation to new tissue formation (Clark, 1996).

3.2. Reepithelialization and new tissue formation

Reepithelialization of the wound begins after keratinocytes at the wound margins become activated for migration. Although the phenotype of these cells begins to change within hours of the injury, keratinocyte migration does not begin in mammals until $\sim$ 24 h postinjury (Woodley, 1996). Dissolution of hemidesmosomes between the basement membrane and basal epithelial cells is a prerequisite to migration along with the formation of peripheral cytoplasmic actin filaments. Following detachment from the basement membrane, epidermal cells at the wound edge extend lamellipodia into the wound. The migrating layer of cells expresses keratins normally found only in the basal cells of the epidermis and pass through the blood clot generating collagenase and plasminogen activator (Clark, 1996). Some days after injury, epithelial cells at the wound margin begin to proliferate under the influence of locally generated growth factors including EGF, TGF- α , and FGFs originating from keratinocytes, macrophages, and dermal fibroblasts (Woodley, 1996). After reepithelialization is complete, a new basement membrane is synthesized from the wound margin inward and epidermal cells renew their attachment to the new basement membrane via hemidesmosomes. During reepithelialization, stem cells from the hair bulge also contribute to this migratory wave of cells, and while these cells are initially found within the neoepidermis, their presence is transitory and they usually disappear within 3–4 weeks (Ito et al., 2005).

Approximately, 4 days after injury, new tissue begins to form in the wound bed consisting of capillaries, macrophages, fibroblasts, and connective tissue, and this fibrous tissue is commonly referred to as granulation tissue (Fig. 4.1). Cytokines and growth factors stimulate angiogenesis,

fibroblast proliferation, and ECM production as the fibrin clot, which consists of collagen, fibronectin, and hyaluronic acid (HA), provides a scaffold for this cellular invasion. Growth factors such as PDGF and TGF β 1 stimulate fibroblasts to migrate via the upregulation of integrin receptors, and in order to move through the fibrin clot, matrix metalloproteinases (MMPs) are produced to cleave a path for this invasion (Mignatti et al., 1996). Once fibroblast migration is completed, some fibroblasts are activated to become myofibroblasts and switch to a synthetic function, depositing a loose network of fibers consisting primarily of fibronectin, collagen types I, III, and VI.

3.3. Tissue remodeling

In the second and third weeks of healing, myofibroblasts expressing α -smooth muscle actin utilize the newly deposited ECM to contract the wound contributing to its closing. During this period, granulation tissue begins the remodeling process and will eventually become mature scar tissue. This process is dependent on the balance between collagen synthesis and collagen catabolism by MMPs. HA and fibronectin disappear, collagen type I bundles enlarge increasing the tensile strength of the wound, proteoglycans are deposited, and slowly the scar tissue becomes relatively acellular. Ultimately, the scar that forms is identified morphologically by thick bundles of collagen running parallel to the epidermis atop the wound bed compared to the “basketweave” pattern of the surrounding uninjured dermis. This new scar tissue usually attains a tensile strength only 70% as strong as intact skin. As outlined by Billingham and Russell (1956a), a wound can only be considered “complete” insofar as the wound ceases to contract.



4. INSIGHTS FROM FETAL WOUND HEALING

Although adult mammalian skin usually heals with a scar following full-thickness excisional or incisional wounding, there is one situation where similar wounds heal in a scar-free manner, namely, in the fetus. This situation provides an opportunity to determine the nature of the essential differences between scarring and perfect healing in the same organism, albeit at different developmental stages and states of cellular differentiation. In general, up to the middle of the third trimester of intrauterine gestation, wounds made in mammalian fetuses (mice, rats, rabbits, sheep, pigs, marsupials, monkeys) heal perfectly (Ferguson and O’Kane, 2004; Larson et al., 2010; Lo et al., 2012; Lorenz and Adzick, 1993; Satish and Kathju,

2010). The precise time at which the transition from scarless healing to scarring occurs in the embryo depends on the size of the wound: larger wounds and excisional wounds (compared to incisional) require earlier gestational periods to heal perfectly (Cass et al., 1997), but for any consistent wound, there is a clear transition period. For example, using 1-mm excisional wounds in the mouse, E17 wounds heal perfectly and regenerate hair follicles, whereas E19 wounds form scars and do not regenerate hair follicles (Colwell et al., 2006).

Following the initial report of human fetuses healing amputation wounds without an inflammatory reaction or granulation tissue formation (Rowlatt, 1979), it was suggested that the sterile, aqueous environment of the uterus was important. However, subsequent studies on marsupial embryos, which leave the uterus early in development to continue in the nonsterile, aerobic conditions of the pouch, still heal perfectly with no scars (Armstrong and Ferguson, 1995) demonstrating that the sterility and chemistry of amniotic fluid are not responsible for scar-free healing. In support of this conclusion, adult sheepskin grafted to an embryo heals with a scar (Longaker et al., 1994). Clearly, this is not an environmental phenomenon but more likely to be an intrinsic property of the skin itself because later embryos (e.g., E19 mouse embryos) are still in the uterus when they heal with a scar. The essential differences borne out of studies comparing fetal and adult wound healing are that fetal wound healing is characterized by (1) minimal inflammation, (2) reduced fibroplasia, and (3) less neovascularization. In addition, as new ECM is deposited in the wound bed, the timing of its appearance and the components of the ECM appear to be different (Kathju et al., 2012; Larson et al., 2010; Lo et al., 2012; Satish and Kathju, 2010).

4.1. Inflammation

The fetus has a less developed immune system, and a blunted inflammatory response can readily be detected: an embryonic wound has fewer neutrophils, lymphocytes, monocytes, and macrophages, and the length of time inflammatory cells are present at the wound site is markedly reduced (Cowin et al., 1998). If a reduced inflammatory response is important for scar-free healing, then regenerating animals should display this phenomenon and this is in part true (see below). However, reducing neutrophil numbers (and thereby reducing early inflammation) in adult mammals has not led to a regenerative outcome since depletion of neutrophils has little effect on

healing and scarring of aseptic wounds (Simpson and Ross, 1971, 1972), despite an increased rate of reepithelialization (Dovi et al., 2003). In addition, 1-day old PU.1 knockout mice which are born without macrophages and functioning neutrophils heal wounds and scar similarly to their wild-type counterparts despite reduction in IL6 and TGF β 1 levels (Martin et al., 2003). The converse, however, certainly is the case because an excessive or persistent neutrophilic inflammatory response in the adult can lead to poor wound healing and excess fibrosis (Satish and Kathju, 2010), and an adult inflammatory response induced in the fetus by the subcutaneous implantation of bacteria increased collagen deposition, fibroplasia, and neovascularization, all characteristics of adult scarring wounds (Frantz et al., 1993). As in the case of extended neutrophil response, a persistent macrophage response can lead to excess scar formation (Satish and Kathju, 2010). Thus, too much inflammation has a negative influence on healing, while decreasing inflammation does not induce regeneration.

4.2. Cytokines

What about the cytokines that these inflammatory cells bring to the wound: if the inflammatory response is less intense in the fetus, then we would expect lower levels of proinflammatory molecules or the receptors they activate? The specific cytokines released by platelets and leucocytes all follow this general theme—low or absent in the fetal wound and application in excess induces an adult, scarring response. PDGF from platelets, for example, is detectable in fetal wounds (Whitby and Ferguson, 1991a) where it is in lower concentrations than in adults (Olutoye et al., 1996). PDGF is a chemotactic agent for inflammatory cells and fibroblasts, and it induces the myofibroblast phenotype along with collagen and HA deposition (Haynes et al., 1994). However, lower levels of PDGF in the adult have been associated with a failure for reepithelialization to occur, such as in nonhealing ulcers and diabetic mice (Beer et al., 1997; Pierce et al., 1995), suggesting that certain levels of PDGF are required for healing. Indeed PDGF is used clinically as an agent for chronic ulcerations (Mustoe et al., 1994) and so reduced levels are unlikely to have a beneficial role in scarring.

VEGF is produced by a range of cells—epithelial, endothelial, leukocytes, fibroblasts—and is crucial for angiogenesis as the wound bed matrix becomes revascularized (Clark, 1996). As there is less neovascularization in fetal wounds, then accordingly, there are as expected, lower levels of VEGF compared to adult wounds. Moreover, in late gestation stages where

fetal wounds heal by scarring, VEGF levels and vascularization increase (Wilgus et al., 2008). However, conflicting results have been obtained showing the opposite pattern of VEGF mRNA levels with increased expression after scarless healing compared to scarring (Colwell et al., 2005). Nevertheless, the application of VEGF protein to nonscarring fetal wounds increases vascularization, collagen deposition, fibroblast number, myofibroblast activation and ultimately can produce a scar. Conversely, application of anti-VEGF antibodies to adult wounds has been shown to decrease the width of the wound by nearly 75% (Wilgus et al., 2008).

There are clear differences in interleukin production between fetal and adult wounds. IL-6 is a proinflammatory cytokine whose expression is far lower in human fetal fibroblasts than in adult fibroblasts, and the response of adult wounds to this signaling molecule continues for an extended period of time (Liechty et al., 2000). Supporting the importance of this particular cytokine, when IL-6 is added to the skin of the nonscarring fetal wounds, scarring occurs. IL-8, another proinflammatory cytokine, is produced by a variety of cell types in the wound, primarily suprabasal keratinocytes, and its production in fetal wounds is lower than in the adult (Satish and Kathju, 2010). It has been shown to increase keratinocyte proliferation and enhance reepithelialization and higher levels than normal are associated with non-healing diabetic wounds suggesting that the primary effect of this cytokine is upon the epidermis (Rennekampff et al., 2000). The same effect applies to TNF- α and IL-1 which are produced from leucocytes in the wound bed and by keratinocytes. At low levels, these molecules appear to stimulate normal reepithelialization, but in chronic wounds, their levels are elevated thereby slowing the rate of reepithelialization (Barrientos et al., 2008).

On the other hand, IL-10 is a potent antiinflammatory cytokine whose levels are high in fetal wounds and serves to inhibit the expression of IL-6 and IL-8 (Liechty et al., 2000). In IL-10-deficient fetal mice, the wounded skin displays an abnormal cellular inflammatory response, increased and abnormal collagen deposition, and evidence of scarring. Conversely, IL-10 overexpression in adult excisional wounds appears to restore normal dermal architecture and hair follicles in the wound bed through efficient reduction of inflammatory cell infiltrate, IL-6 production and levels of the profibrotic mediators HSP47 and MCP-1 (Peranteau et al., 2008).

Among all cytokines characterized during the repair process, TGF β -signaling members play a pivotal role through regulating inflammation, fibrosis, and scarring (Werner and Grose, 2003). TGF- β 1 is released by platelets and is chemotactic for neutrophils, monocytes, and fibroblasts,

which in turn can synthesize additional TGF- β 1 and TGF- β 2 (Barrientos et al., 2008). TGF- β 1 and TGF- β 2 (from platelets and macrophages) are minimally produced in fetal serum (Olutoye et al., 1996) and at minimal or lower levels in fetal wound tissue compared to adults (Cowin et al., 2001; Ferguson and O'Kane, 2004; Sullivan et al., 1995). The addition of excess TGF β 1 induces fibroplasia, deposition of collagen, and scarring in human and rabbit fetal wounds (Krummel et al., 1988; Sullivan et al., 1995). Conversely, the application of isoform-specific anti- β -1 and β -2 antibodies to adult incisional wounds resulted in less vascularization, decreased inflammatory response, less collagen deposition, more normal collagen fiber orientation, and there was a marked reduction in visible scarring (Shah et al., 1995). The third isoform, TGF- β 3, is present at high levels in fetal scarless wounds and at low levels in adult wounds. The positive role of TGF- β 3 in inhibiting scarring is revealed in studies of the TGF- β 3null mouse whose embryos show scarring, and in the adult wound, the application of excess TGF- β 3 reduces scarring (Ferguson and O'Kane, 2004; Occleston et al., 2008; Proetzel et al., 1995; Shah et al., 1995).

Other growth factors, including FGF family members, show complex patterns during scar-free healing and scarring in the fetus because of the existence of multiple isoforms and multiple receptors. In general, however, there seems to be an overall downregulation of FGF signaling during scarless healing (Dang et al., 2003a), although others have reported higher levels of FGFs by immunocytochemistry in the fetus (Whitby and Ferguson, 1991a).

4.3. ECM composition

Fetal skin is still developing and far from mature. Not surprisingly, the ECM shows considerable structural differences between fetal and adult skin. The collagen profile is different with fetal skin containing a greater proportion of type III collagen and adult skin containing predominantly type I collagen (Lo et al., 2012). In the fetal wound, collagen types I and III fibers regenerate in a reticular pattern, whereas the adult wound contains densely arranged parallel collagen bundles that are also more highly cross-linked (Cuttle et al., 2005). Correlated with these differences in collagen types is the observation that the cell surface discoid domain receptor (DDR-1) to which collagen fibers can bind is expressed at high levels in early gestation fetal fibroblasts and the levels decrease with gestational age (Chin et al., 2001). This means this tyrosine kinase receptor could play a role in regulating collagen production and organization to prevent scarring.

There are comparatively higher levels (threefold) of glycosaminoglycans (GAGs) in the fetal wound bed, and the compliment of GAG matrix is composed predominantly of HA (DePalma et al., 1989; Krummel et al., 1987). During fetal scarless healing, HA levels increase over normal levels and remain high for 3 weeks, whereas in the adults, they return to normal levels after only 7 days (Longaker et al., 1991). HA thus has a positive influence on regeneration and stimulates protein synthesis of fibroblasts to produce both collagens and noncollagenous proteins (Mast et al., 1993). A reduction in the HA content of fetal rabbit wounds by enzyme action results in increased fibroblast infiltration, collagen deposition, and capillary formation, that is, an adult-like response (Mast et al., 1992). There are higher fibronectin and chondroitin sulfate levels in unwounded fetal skin and fetal wounds, and these structural proteins may be involved along with HA in organizing collagen fibrillogenesis to resemble that of the fetus rather than the adult (Coolen et al., 2010; Whitby and Ferguson, 1991b). The same is true of tenascin-C, which is rapidly upregulated early during wound healing and produced at higher concentrations in fetal wounds (Whitby and Ferguson, 1991b; Whitby et al., 1991).

The enzymes responsible for breakdown and reorganization of the ECM are MMPs and tissue inhibitors of metalloproteinases (TIMPs), and the balance of their activity differs significantly between fetal and adult wounds (Gill and Parks, 2008). MMP1 (a collagenase that breaks down collagen types I and III) and MMP9 (gelatinase that primarily breaks down collagen type IV) both increase following injury in scarless (E16 rat embryos) and scarring embryos (E19 rat embryos), but the increase is more rapid and higher during scarless healing (Dang et al., 2003b). MMP2 (degrades basement membrane proteins) levels appear unchanged during scarless healing, but decrease in adult wounds, whereas TIMPs 1 and 3 are highly elevated in adult wounds. Overall, the current picture is that fetal wounds upregulate MMPs to a higher degree relative to TIMPs when compared to adult wounds that scar.

4.4. Fetal dermal fibroblasts

Apart from quantitative differences among inflammatory cell numbers and the cytokines that they release, it is also possible that there are intrinsic differences in the properties of fibroblasts from fetal and adult wounds, particularly since they are responsible for secreting the ECM in response to a panoply of cytokines. As described above, many fibroblasts migrate into the granulation tissue, transform into myofibroblasts, and participate in

wound contraction. In addition to their contractile function, myofibroblasts, at least in part, are responsible for depositing ECM that contributes to scarring (Desmouliere et al., 2005). For example, fetal sheep wounds that heal scar free have no myofibroblasts within the wound, but as wounds are made on progressively older fetuses, myofibroblasts become present and this is associated with scarring (Estes et al., 1994). In fact, because TGF β 1 application to fetal wounds induces myofibroblast transformation, it is likely that the control of TGF β isoforms plays a role in controlling this event (Lanning et al., 2000). Furthermore, scarring and nonscarring fibroblasts from different aged fetuses respond differently to the same environment. When skin from 15–22 week human fetuses is grafted to a subcutaneous site in adult nude mice, the fetal skin heals without a scar following 5-mm incisional wounding. However, when grafted to a cutaneous site, the same tissues scar (Lorenz et al., 1992). What is the difference between these two situations? When the results were analyzed with species-specific antibodies to collagen, it was found that in the scarless situation (subcutaneous), the collagens laid down were of human (graft) origin despite being in the adult, scarring environment of serum and inflammatory cells. Conversely, in the scarring situation (cutaneous), healing occurred with the deposition of mouse collagen (Lorenz et al., 1995). Thus, there are intrinsic properties of fibroblasts which allow them to respond differently to the same cytokine environment. Furthermore, these properties are retained in culture such as the ratio between collagen types I and III synthesis in response to TGF- β 1 in fetal versus older fibroblasts (Carter et al., 2009), differences which reflect those seen in the wound bed *in vivo* (Goldberg et al., 2007).



5. LIMITATIONS OF EXTRAPOLATING FROM FETAL WOUND-HEALING STUDIES

While the precise mechanisms that regulate scarless healing in fetal mammals remain unknown, there are clear cellular and molecular differences between the fetus and adult as described above. The important question is whether these studies have helped us understand the underlying mechanisms that resolve a wound by regenerating the missing tissue rather than producing a scar? When testing potential candidate molecules as anti-scarring agents based on fetal wound-healing studies, only a handful fulfill the criteria of reducing scarring in adult wounds and none have proven successful at regenerating skin. The molecules that have held the highest potential tend to be specific inflammatory mediators. One candidate is VEGF,

since anti-VEGF antibodies were found to decrease scarring in adults (Wilgus et al., 2008). However, anti-VEGF drugs such as bevacizumab actually inhibit wound healing, suggesting a more complex role for VEGF (Sharma and Marcus, 2013). Another is IL-10, since IL-10-deficient embryos were found to form scars and the overexpression of IL-10 in adults allowed the restoration of normal dermal architecture and hair follicles (Peranteau et al., 2008). These findings led to the development of Prevascar, human recombinant IL-10 which initially showed promise at reducing scarring in a double-blind randomized clinical trial, but further trials are now halted (www.renovo.com). Third, TGF- β 1 and TGF- β 2 induce myofibroblast differentiation, collagen deposition, and scarring, and antibodies to these proinflammatory molecules reduce scarring (Shah et al., 1995). Fourth, TGF- β 3 reduces scarring and its absence increases scarring which led to the development of Avotermin as a potential antiscarring therapy (Ocellston et al., 2011). Although promising, Avotermin, which passed through Phase II clinical trials, was terminated during Phase III trials because it failed to meet its primary endpoint (www.renovo.com).

These examples underscore how difficult it has been to develop effective human therapies based on fetal wound-healing studies. It is conceivable that misleading information has been obtained by comparing the fetal and adult wound-healing scenarios because of the completely different differentiation state of the cells and the skin. Fetal skin is still progressing through its final stages of embryonic development with a large number of fibroblasts still proliferating, along with continued maturation of the dermis and associated hair follicles. Damage at this stage may not have to reactivate genetic programs to repair the damage and instead, the excised tissue may simply “carry on” developing, which is interpreted as scar-free healing. Adult tissues, however, are fully differentiated, exhibit little proliferative activity, and have to first dedifferentiate (lose their differentiated phenotype and reenter the cell cycle) in order to respond to the damage. It may be that the ability of cells to dedifferentiate and return to a fetal-like genetic program is lacking in a scarring mammalian wound response, and so by comparing adult wounds with fetal wounds, we would not generate any insights into this process. In contrast, comparing *adult* wounds in animals that heal scar free with mammalian wounds that scar may produce new insights particularly with regard to the local environment and how it might relate to dedifferentiation. We suggest that there is a need to explore skin repair more broadly across *regenerating adult* vertebrate models for their potential to contribute to the wound-healing field and present examples below.



6. SKIN REGENERATION IN ADULT VERTEBRATES

Regeneration research over the past 100 years has made it clear that all vertebrates, including mammals, possess some ability to undergo reparative regeneration of complex tissue structures (Vorontsova and Liosner, 1960), although the regenerative ability of some vertebrate organ systems appears to be developmentally constrained (Seifert and Voss, 2013). Despite the fact that the skin is the largest vertebrate organ and the first line of defense against external injury, it is a surprisingly understudied organ system with respect to vertebrate wound healing, mammals not withstanding. Wound-healing studies, in fact, have for the most part almost exclusively focused on laboratory and domestic mammals. Recently, investigations into the mechanisms underlying skin repair and regeneration in other vertebrate animal models have been initiated with the specific intention of seeking comparative information for how different vertebrates repair skin injuries (Bertolotti et al., 2013; Guerra et al., 2008; Levesque et al., 2010; Richardson et al., 2013; Seifert et al., 2012a,c; Yannas et al., 1996; Yokoyama et al., 2011).

Comparative anatomy suggests that the basic structure of full-thickness adult skin is conserved across aquatic and terrestrial vertebrates, although the protective function of the epidermis diverges along two developmental trajectories; keratinization and mucogenesis (Fig. 4.2; Henrikson and Gedeon Matoltsy, 1968). Fishes and aquatic amphibians possess a stratified squamous epithelium that lacks a stratum corneum and is populated by a number of specific cell types necessary for mucous production and immunity (Dawson, 1920; Flaxman, 1972; Hawkes, 1974; Henrikson and Gedeon Matoltsy, 1967; Seifert et al., 2012c; Fig. 4.2A and D). Terrestrial vertebrates possess a more highly stratified epidermis, and cell differentiation occurs as cells move from the stratum germinativum toward the outer surface (Fig. 4.2B and C). In most vertebrates, the dermis is largely subdivided into two layers, although the dermis is greatly reduced in zebrafish. The uppermost layer, the stratum spongiosum or papillary dermis, is in tight contact with the basement membrane of the epidermis and consists of loosely assembled collagen and elastin fibers populated by fibroblasts, pigment cells, and immune-surveillance cells (Fig. 4.2D–F). Beneath this layer, the stratum compactum or reticular dermis provides tensile strength to the skin through a vast network of collagen and elastic fibers in association with nonfibrous ECM molecules. Beneath the dermis is a layer of subcutaneous tissue composed of adipose cells and ECM called the hypodermis, and this separates the

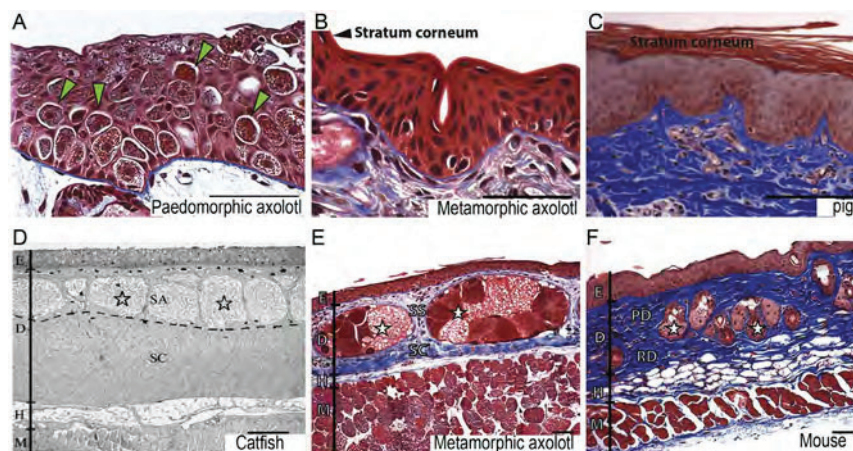


Figure 4.2 Comparative anatomy of vertebrate skin. (A) Paedomorphic axolotl epidermis (*Ambystoma mexicanum*). Representative of mucogenic epidermis present in most cartilaginous and bony fish, larval and neotenic amphibians. Green arrows indicate Leydig cells that are highly granulated. (B) Metamorphic axolotl (*Ambystoma mexicanum*). (C) Pig (*Sus scrofa*). Both B and C represent keratinized epidermis typical of terrestrial vertebrates. A layer of dead cells, the stratum corneum, covers the epidermis and is more prevalent in mammals. (D) African catfish (*Clarias gariepinus*) from Guerra et al. (2008). (E) Metamorphic axolotl (*Ambystoma mexicanum*). (F) Mouse (*Mus*). (D–F) Show full-thickness skin with generally similar cellular organization. The dermis contains appendages such as granular and mucous glands, scales, hair follicles, sebaceous, and sweat glands (stars). The dermis is subdivided into an upper layer (SA, SS, stratum spongiosum; PD, papillary dermis) and lower layer (SC, stratum compactum; RD, reticular dermis). Beneath the dermis lies a layer of adipose tissue termed the hypodermis (H). M, muscle. Scaled fish skin (not pictured) does not possess a well-defined dermal layer.

skin from the underlying muscle. Dermal glands, hair follicles, and feathers all derive from epidermal precursors, whereas scales are of mesenchymal origin, and all of these structures are embedded within the fibrous network of the dermis (Fig. 4.2, stars). With this in mind, the Sections 6.1–6.3 explore how a variety of animal models are contributing insight into the overall process of skin regeneration.

6.1. Cartilaginous and bony fishes

With the caveat that the rate of wound healing in cold-blooded animals is largely dependent on temperature, the few published studies investigating full-thickness wound healing in cartilaginous (sharks, skates, rays) and bony

(ray finned and lobe finned) fishes support the notion that the same general processes observed during mammalian wound repair also occur in these animals (da Silva et al., 2004, 2005; Guerra et al., 2008; Penczak, 1960). Furthermore, given extended observation periods postwounding, minnows (Sauter, 1934), three-spined sticklebacks (Penczak, 1960, 1961), goldfish (Yamada, 1964), zebrafish (Richardson et al., 2013), and sharks (Reif, 1978) demonstrate the ability to regenerate epidermis, dermis, scales, hypodermis, and muscle following full-thickness excisional wounding. In contrast, one study detailing wound healing in Antarctic rockcod found persistence of scar tissue in the wound bed after 90 days and failure to regenerate scales, although it is likely that the near freezing temperatures these animals reside at (0 °C) could affect the timeline of healing (da Silva et al., 2004, 2005). Nevertheless, viewed collectively, a general set of processes can be gleaned from these studies with the majority of interspecific variation affecting the *rate* of healing in excisional wounds.

Following skin excision, a hemostatic response occurs and bleeding ceases either by formation of a wet scab (Sauter, 1934) or through coagulation of mucous and blood at the wound site (da Silva et al., 2004, 2005; Guerra et al., 2008). An edema is usually observed in the wound bed, and this substrate, along with the underlying muscle, provides a matrix over which keratinocytes can migrate directly, as opposed to through. The available evidence suggests that migration of keratinocytes from the free wound edges begins rapidly, although there is apparently a high degree of interspecific variation. In African catfish, hemidesmosomal attachment of the epidermis to the basement membrane is released within the first 3 h after injury (Guerra et al., 2008) and in minnows keratinocytes began migrating within 12–24 h of injury (Sauter, 1934). Once migration ensues, it progresses rapidly and large wounds can reepithelialize in as little as 24 h in African catfish (Guerra et al., 2008) or between 2 and 5 days in minnows (Sauter, 1934) and sticklebacks (Penczak, 1960, 1961). Adult zebrafish can completely reepithelialize 2-mm wounds in 7 h, while reconstituting a stratified epithelium in 24 h (Richardson et al., 2013). Conversely, epidermal migration in Antarctic rockcod does not begin until 1 week postinjury, and these wounds take between 3 and 4 weeks to reepithelialize (da Silva et al., 2004). However, time to reepithelialization appears to be independent of regenerative ability as large wounds in leopard sharks take between 2 and 3 weeks to reepithelialize, and these animals regenerate full-thickness skin and scales (Reif, 1978).

These studies documenting skin regeneration also support activation of an inflammatory phase shortly after skin injury. During wounding in

minnows, inflammatory cells are observed to infiltrate into the wound bed (Sauter, 1934), a process which peaks at 3 days postinjury (dpi) in African catfish (Guerra et al., 2008). Despite low temperatures, the inflammatory profile in Antarctic rockcod mimics that seen during mammalian wound healing, with an initial influx of neutrophils that are slowly replaced by macrophages and lymphocytes (da Silva et al., 2005). Interestingly, this study demonstrated the persistence of macrophages in the wound bed as far out as 90 dpi, and the authors suggested that the failure of these wounds to regenerate scales might result from an extended inflammatory phase. Unfortunately, these studies did not quantify the strength of the inflammatory response, either by cell counting or examination of pro- and antiinflammatory cytokine profiles in the wound bed. The inflammatory response was similarly found to be strong shortly after wounding in zebrafish, with neutrophils and macrophages peaking in the wound bed 8 h postinjury and returning to baseline levels by 6 dpi (Richardson et al., 2013).

Following hemostasis, inflammation, and reepithelialization, fibrosis was observed to occur throughout the wound bed in several species (da Silva et al., 2004; Penczak, 1960). In African catfish lacking scales, the stratum spongiosum was completely regenerated in 28 days from fibrous tissue deposited in the wound bed, although the unique adipose structures in this dermal layer were smaller than their unwounded counterparts (Guerra et al., 2006, 2008). In scaled fish, production of fibrous tissue throughout the wound bed precedes initiation of scale regeneration, and by 30 dpi, this matrix is remodeled during regeneration of the dermis (Penczak, 1960, 1961). Lateral plate regeneration in sticklebacks initiates around 50 dpi and after 100–150 days the original plates are restored, although sometimes they are of irregular shape and size (Penczak, 1961). The process of scale regeneration in other species appears similar, as minnows, zebrafish, sharks, and goldfish all regenerate scales from within the wound bed between 28 and 100 dpi (Reif, 1978; Richardson et al., 2013; Sauter, 1934; Yamada, 1964). Of interest is that regenerated scales are sometimes of different size and orientation relative to scales at the wound margins suggesting loss of patterning information relative to the surrounding scales (Fig. 4.3A–C). Lastly, it is important to note that unlike studies investigating scale regeneration where individual scales are removed from their scale pockets, these studies completely excised all dermal tissue, and thus new scales were regenerated not from a piece of existing scale, but instead from an unknown cellular source within the wound bed. This fact argues for *de*

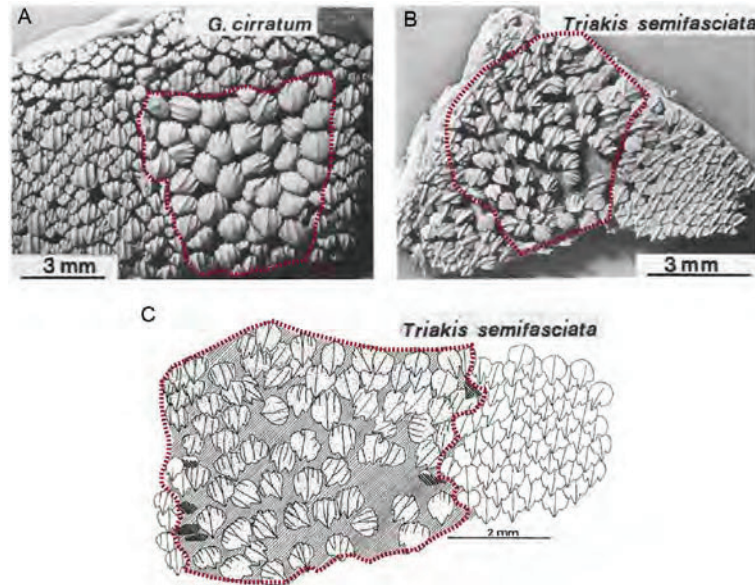


Figure 4.3 Scale regeneration 4 months after full-thickness skin injury in a nurse shark (*Ginglymostoma cirratum*) and a Leopard shark (*Triakis semifasciata*). (A–C) Red outline represents the injury area. (A) Regenerated scales in the nurse shark are larger and orientated at varying degrees compared to the smaller, uniformly orientated uninjured scales. (B–C) Regenerated scales in the leopard shark display a similar pattern to A, with fewer scales in the regenerated area. Adapted from Reif (1978).

*nov*o development of these scales independent of the surrounding scale pattern and leaves open the question of how these scales are induced to form. Taken together, these findings support the idea that adult cartilaginous and bony fish are capable of regenerating full-thickness skin following complete excision, and they provide a detailed account of the basic cellular processes involved. These studies furnish us with a sound foundation to continue exploring wound healing in fishes and with the emergence of the zebrafish as a tractable genetic model for developmental studies, create an enormous potential for further investigation.

6.2. Anurans and urodeles

Amphibians have been useful animal models to study the mechanisms that regulate reparative regeneration because they display a range of regenerative abilities and are tetrapods. Their phylogenetic position relative to mammals

and their similar musculoskeletal structure also makes them better suited for comparative analysis. Anurans (frogs, toads) exhibit a stage-dependent loss of regenerative ability approaching metamorphic climax (Dent, 1962; Forsyth, 1946; Muneoka et al., 1986; Vorontsova and Liosner, 1960) and as such, exhibit poor regenerative abilities as adults. During this transition, there is a proximal to distal loss of regenerative ability in the limb that is correlated with transformation of larval skin to a terrestrial form, formation of striated muscle, and differentiation of the skeleton (Forsyth, 1946; Korneluk and Liversage, 1984; Rose, 1944; Wolfe et al., 2000). In contrast, urodeles (salamanders and newts) have long been considered masters of vertebrate regeneration and exhibit the ability to regenerate limbs, tails, spinal cord, jaws, brain, and lens as adults. Thus, it is all the more surprising that relatively few investigations have utilized these animal models to directly examine how skin wounds repair and regenerate (Baitsell, 1916; Endo et al., 2004; Levesque et al., 2010; Loeb and Strong, 1904; Seifert et al., 2012c; Suzuki et al., 2005, 2007; Yannas et al., 1996; Yokoyama et al., 2011). More recently, studies have examined flank skin wounds outside of regeneration fields like the limb and tail with the explicit purpose of contextualizing the findings within the mammalian wound-healing literature (Bertolotti et al., 2013; Seifert et al., 2012c; Yokoyama et al., 2011).

Because regenerative ability of the limb in anurans progressively declines during metamorphosis, the *a priori* hypothesis for skin repair would be that larval individuals would regenerate skin while adults would heal by scarring. This would echo the situation in mammals when comparing fetal and adult wound healing. The few wound-healing studies performed in anurans allow us to at least partially reject this hypothesis because some degree of regeneration is seen across developmental stage and species (Bertolotti et al., 2013; Suzuki et al., 2005; Yannas et al., 1996; Yokoyama et al., 2011), although differences in wound type (excisional versus incisional) and wound site (limb versus flank) appear to affect the outcome.

Examining the early phases of wound healing after full-thickness excisional flank wounding in adult frogs, blood and lymph are observed to clot within minutes to achieve hemostasis and reepithelialization of 5×2 mm wounds is completed in 48 h (Baitsell, 1916). *Xenopus* froglets (which are juvenile animals) reepithelialize 1×1.5 mm excisional flank wounds in 24 h (Yokoyama et al., 2011), whereas reepithelialization of incisional wounds on the hindlimbs of adult *Xenopus laevis* is age dependent, with 8-month-old adults covering the wound bed in 24 h and 15-month adults taking 3 days (Bertolotti et al., 2013). The inflammatory response is activated

within 24 h in adult *Xenopus*. Neutrophils, macrophages, and lymphocytes are found throughout the wound bed at 7 dpi, and these cells are immunoreactive for TNF α , iNOS, TGF β 1, and MMP-9 (Bertolotti et al., 2013). Although a detailed analysis was not performed in *Xenopus* froglets, inflammatory cells were not reported in the wound bed of juvenile animals suggesting *Xenopus* froglets are more similar to fetal mammals rather than adults when it comes to wound healing (Yokoyama et al., 2011).

The most consistent observations across anuran wound-healing studies are the characterization of new tissue formation in the wound bed, remodeling of the new ECM into dermis, and regeneration of exocrine glands. Regardless of developmental stage or species, a fibrin clot is observed in both excisional and incisional wounds by 3 dpi and within 1 week fibrous tissue is found throughout the wound bed (Baitsell, 1916; Bertolotti et al., 2013; Yannas et al., 1996; Yokoyama et al., 2011). This fibrous tissue bears a strong similarity to granulation tissue formed in mammalian wounds. This granulation tissue exhibits angiogenesis, and myofibroblasts are found throughout the tissue as indicated by α -smooth muscle actin localization in fibroblastic cells (Bertolotti et al., 2013). Following deposition of fibrous tissue throughout the wound bed in adults, however, the remodeling process does not appear to lead to complete dermal regeneration (Bertolotti et al., 2013; Yannas et al., 1996).

This interpretation is based partially on the observation in adult *Lithobates catesbeiana* (American bullfrog) that new regenerated glands in the wound bed lack ducts, and thus functionality (Yannas et al., 1996). However, our wound-healing data in *Lithobates sphenoccephalus* (southern leopard frog) and *Xenopus mulleri* support the notion that anurans are capable of regenerating glands with exocrine ducts suggesting they are indeed functional (Fig. 4.4A and B). In agreement with our data, regenerated glands in adult *X. laevis* possess intact ducts supporting the functional replacement of new glands (Bertolotti et al., 2013). While it seems clear that wounded skin can regenerate glands, of interest is the quality of dermal regeneration that accompanies regeneration of the epidermis and associated glands. The extent of dermal repair in adult *L. catesbeiana* and *X. laevis* was considered as an amphibian scar based on (1) the lack of a completely defined stratum compactum with irregular structural features, (2) increased thickness of the dermal tissue, and (3) persistence of dense fibrous tissue in the wound bed (Bertolotti et al., 2013; Yannas et al., 1996). However, since new glands regenerated along with the stratum spongiosum, another interpretation of these results is that both regeneration and scarring occurred. In support of

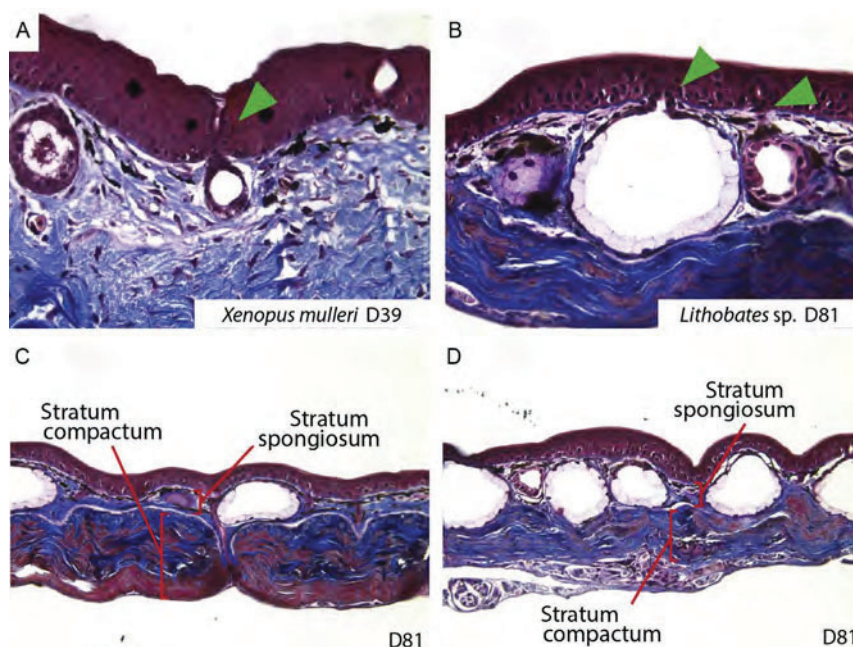


Figure 4.4 Skin regeneration and scarring in anurans. (A and B) Exocrine gland regeneration in *Xenopus mulleri* 39 days postinjury (dpi) and in *Lithobates* sp. 81 dpi. Exocrine ducts are visible from the regenerating glands (green arrows). (C and D) Full-thickness skin regeneration in *Lithobates* sp. after 81 days, (D) exhibits some slight alterations to the structure of the stratum compactum compared to unwounded skin from the same animal adjacent to the wound (C). New glands are also present throughout the wound bed of the regenerated skin (D).

this notion, our data from 4-mm full-thickness excisional wounds in adult *L. sphenoccephalus* suggest that some adult anurnas are capable of adequate structural replacement to consider the result regeneration (Fig. 4.4C and D). While the stratum compactum may lack the perfect structural equivalent of unwounded dermis, stratification of the dermis in conjunction with gland regeneration supports skin regeneration, not scarring (Fig. 4.4D). It is possible that incisional wounding elicits a more severe fibrotic response compared to an excisional wounding and that this in turn affects remodeling as does a potential contribution of age, as this was unknown for adults in these studies. From a clinical standpoint, however, regeneration of hair follicles in mammalian excisional wounds accompanied by disorganized collagen fibers in the dermis would be a welcome achievement in regenerative medicine.

While anurans exhibit progressive loss of regenerative ability approaching metamorphosis, urodeles appear to enjoy lifelong regenerative capacity as adults (Eguchi et al., 2011; Seifert et al., 2012b; Young et al., 1983). Until recently, wound-healing investigations using salamanders and newts focused on the migratory ability of keratinocytes and their preferential use of specific ECM proteins (Donaldson and Dunlap, 1981; Donaldson and Mahan, 1983; Donaldson et al., 1985, 1995; Ferris et al., 2010). An *ex vivo* skin explant system demonstrated nicely how leading edge keratinocytes begin migrating after 1–2 h and require MMP activity for reepithelialization to occur (Ferris et al., 2010). An examination of excisional wounds on the forelimbs of larval axolotls paralleled similar experiments in larval anurans, demonstrating scar-free healing and skin regeneration (Endo et al., 2004; Suzuki et al., 2007), and another study used excisional wounds on the tails of larval axolotls to examine epidermis and basement membrane regeneration (Levesque et al., 2010). Interestingly, although the tail skin lacked a defined dermis and glands had yet to develop, fibrous tissue was observed transiently during regeneration and ultimately was remodeled into a thin layer beneath the epidermis (Levesque et al., 2010).

Building on work in larval salamanders, we recently sought to develop the adult axolotl as a comparative model for adult skin regeneration (Seifert et al., 2012c). Using a 4-mm full-thickness excisional wound model popular in mammalian wound-healing studies, we examined skin regeneration over a 180-day period. Similar to studies described above on *Xenopus* froglets, we found that aquatic axolotls could regenerate their skin, including epidermis, glands, and dermis 80 dpi. Because aquatic axolotls retain certain larval skin features, we compared wound healing between paedomorphic (aquatic) and metamorphic (terrestrial) axolotls that we induced to undergo metamorphosis via thyroxine exposure. Aquatic axolotls have epidermis that is mucogenic and is more similar in form to fish epidermis, while metamorphosis to a terrestrial form elicits transformation of the epidermis to a keratinized epithelium similar to mammalian epidermis (Page et al., 2009; Seifert et al., 2012c; Fig. 4.2A–C). Comparing paedomorphic and metamorphic axolotls, metamorphs exhibited a delay in activation of wound edge keratinocytes and subsequently a prolonged period of reepithelialization, increased influx of inflammatory cells, prolonged period of new ECM production, and an almost doubling in the time required for complete skin regeneration (Seifert et al., 2012c). Although the regenerated glands were smaller than those in unwounded skin and the stratum compactum exhibited slight structural alterations, terrestrial adults were still capable of regenerating full-thickness skin.

While we did not directly quantify and compare inflammatory cell numbers between terrestrial axolotls and mammals during wound healing, it was clear that neutrophil influx was low, and we observed macrophages throughout the wound bed during inflammation. We found migrating epidermis and new epidermis covering the wound bed expressed an MMP profile reminiscent of mammalian wound healing, supporting a role for these molecules across species (Endo et al., 2004; Seifert et al., 2012c). In addition, our transcriptional analysis of the epidermis during the first 7 days of wound healing detected a shift in keratin expression that mirrored that seen in mammals with a conspicuous upregulation of keratin 17 (Ashley Seifert). New ECM was produced between 10 and 14 dpi and persisted throughout the wound bed. Analyzing the composition of this fibrous tissue, we found it contained fibronectin, high levels of tenascin-C, and collagen, with collagen type III produced first and slowly replaced with collagen type I during remodeling. Interestingly, tenascin-C levels remained high throughout the wound bed until the dermis had regenerated. Similar to fishes and anurans, we observed new glands regenerating from the overlying epidermis ~40 dpi as the stratum spongiosum began to reform. Compared to mammals, we found contraction rates similar to tight-skinned mammals (e.g., humans and pigs), but did not observe many myofibroblasts within the fibroblast population of the wound bed.

When fish and amphibian wound-healing studies are compared to mammalian wound repair what emerges is a picture where the general processes described for mammalian wound repair also occur during skin regeneration and instead it is the control of these processes that must be important (Fig. 4.5). First, although a fast rate of reepithelialization is often invoked as a key component of healing outcome and regenerative ability, these data suggest that the ability to regenerate skin is not dependent on the timing of reepithelialization, only that the surface be ultimately reepithelialized. Second, the characterization of *de novo* gland regeneration from the new epidermis overlying the wound bed strongly suggests that the mechanisms underlying embryonic gland development are reactivated during the healing process. Third, dermis and scale regeneration are preceded by formation of fibrous tissue similar to granulation tissue in mammals that fills the wound bed. The regenerated dermis is ultimately remodeled from this fibrous tissue, rather than being assembled *de novo* as in embryonic development (Fig. 4.5). The finding that myofibroblasts are more populous in anuran wounds that produce a greater scarring response compared with urodele skin wounds supports the importance of this fibroblast phenotype as a critical regulator

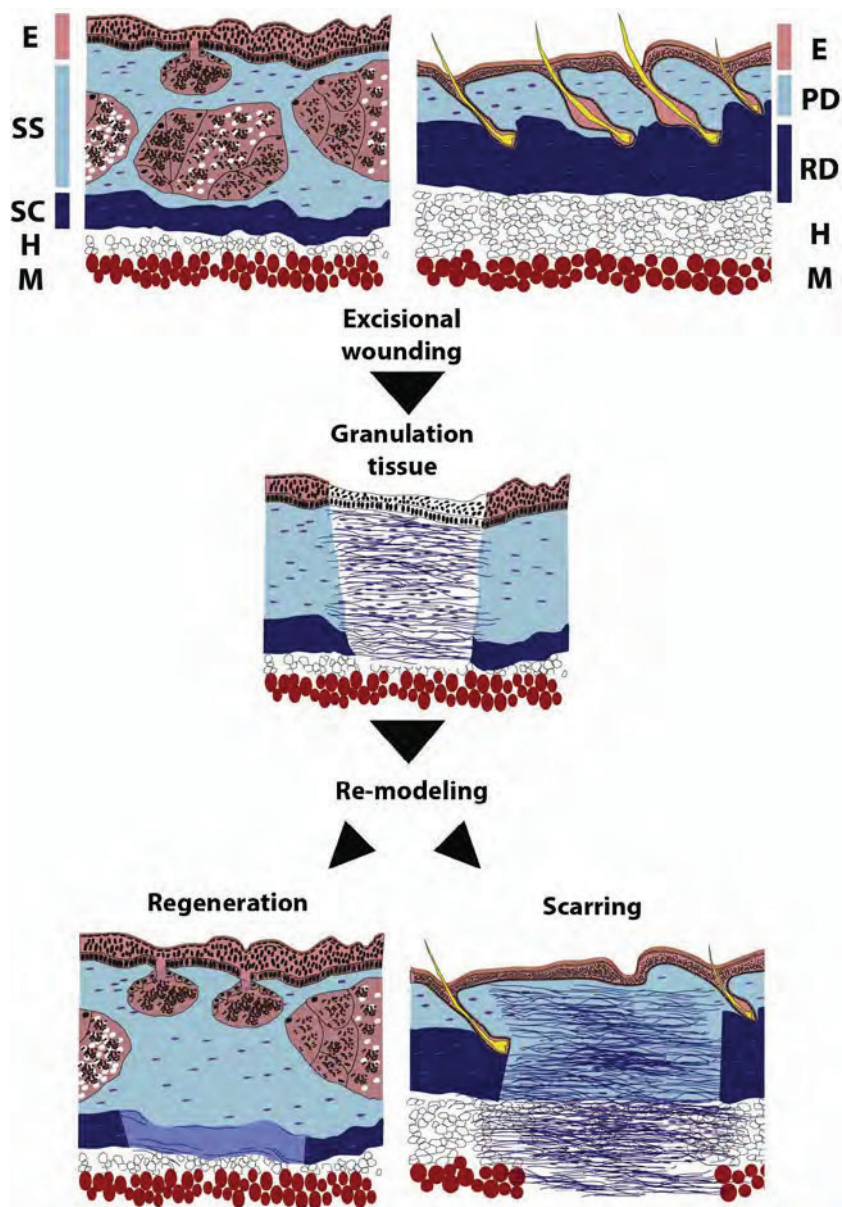


Figure 4.5 Fibrosis precedes both regeneration and scarring in vertebrates. In adult vertebrates where skin regeneration has been described, the wound bed becomes filled with extracellular matrix resembling granulation tissue found in mammalian wounds that scar. Remodeling of this fibrous tissue will lead to restoration of the dermis during regeneration, but will persist as a scar in mammals. Although the reepithelialization occurs in both scarring and regeneration, epidermally derived appendages (e.g., glands and hair) do not regenerate when a scar persists. E, epidermis; SS, stratum spongiosum; SC, stratum compoactum; H, hypodermis; M, muscle.

of regeneration or repair. Importantly, it appears that regeneration of glands accompanies reorganization of the stratum spongiosum suggesting that communication between epidermis and reorganizing dermis is necessary for gland regeneration. Interestingly, the failure of hair follicle regeneration in mammals may result from the intensity of fibrosis occurring throughout the dermis which may impinge on this communication. Lastly, while much has been made of how reduced inflammation can affect the repair process, the participation of major inflammatory cells types during skin regeneration in fishes and amphibians suggests that the cytokine profile of these cells is likely more important than the presence of the cells themselves. In agreement with this idea, a recent study on limb regeneration in axolotls found a complex pattern of both pro- and antiinflammatory molecules during the initial stages of regeneration (Godwin et al., 2013). Furthermore, depletion of macrophages completely inhibited limb regeneration supporting the positive role that inflammatory cells play during the regenerative process (Godwin et al., 2013). When viewed collectively among regeneration studies in other organ systems, the results from skin-healing studies in non-mammalian vertebrates support the hypothesis that epimorphic limb regeneration and skin regeneration proceed through different processes. This comparison underscores the importance of developing wound-healing models across different species that focus directly on skin.

6.3. Mammalian models of adult skin regeneration

While we have argued above that mammalian wound-healing research can benefit immensely from research conducted in nonmammalian vertebrates through the development of adult models of skin regeneration, adult mammalian models of bonafide skin regeneration would represent an excellent tool to interrogate how fibrosis can be controlled and skin injuries stimulated to regenerate. Interestingly, there have been several adult models of mammalian skin regeneration that have been underutilized for wound-healing studies, partly because of logistical concerns, a lack of molecular genetic tools and favoritism toward genetic mouse models with improved healing because of the molecular resources in the laboratory mouse. We discuss some of these models below and their implications for skin regeneration in humans.

As it turns out, there are several instances in which adult mammals can regenerate full-thickness excisional wounds, including epidermal appendages, hair follicles, and sebaceous glands. However, for reasons currently unknown, it appears that this ability is restricted to certain species and is a function of

wound size and wound contraction (Billingham and Russell, 1956a,b; Breedis, 1954; Efimov, 1965a,b, 1969; Ito et al., 2007; Seifert et al., 2012a). These instances include large ear punches made through the ear pinnae of rabbits and African spiny mice, large wounds made on the backs of rabbits, African spiny mice, C57BL/6J, SJL or mixed strains and as a special case, velvet covering deer antlers (Billingham and Russell, 1956a,b; Billingham et al., 1959; Breedis, 1954; Efimov, 1969; Goss and Grimes, 1972; Ito et al., 2007; Joseph and Dyson, 1965, 1966a,b; Seifert et al., 2012a; Vorontsova and Liosner, 1960; Williams-Boyce and Daniel, 1980, 1986).

Although work in rabbits and African spiny mice has shown that ear hole regeneration likely proceeds through blastema formation, it appears that regenerating skin associated with the ears may be similar to that observed in large dorsal skin wounds and thus provides important insight into the mechanisms underlying skin regeneration. As first demonstrated by Markelova (Vorontsova and Liosner, 1960), a 1-cm² hole punched through the ear pinnae of a rabbit will completely regenerate the excised structures including epidermis, dermis, hair follicles, sebaceous glands, and cartilage. In subsequent rabbit studies, these findings were confirmed and extended through a detailed account of the regeneration process and several important features pertaining to the skin can be ascertained (Goss and Grimes, 1972; Joseph and Dyson, 1966a,b; Williams-Boyce and Daniel, 1980). First, although the gap between the wound edges was just a few millimeters, reepithelialization took 7 days to complete and histolysis was evident in the wound bed at this time. Second, approximately 21 dpi, new hair follicles began to form from new epidermis and invaginate into the underlying ECM that was composed of loosely, but irregularly, organized collagen and reticular fibers. A mixture of fibroblasts and undifferentiated, mesenchymal cells populated this loosely organized ECM that was distinctly different from the compact, parallel collagen organization typical of mammalian scarring. Thirty-five days post-injury regenerated follicles were readily visible extending from the epidermis and 42 dpi sebaceous glands could be seen developing in association with these follicles. Lastly, regeneration of the new tissue and skin appeared to be closely associated with the degree of contraction. When ear holes regenerated and new skin formed, contraction was minimal, accounting for only 1–5% of wound area, whereas when contraction accounted for 22–32% of closure, the defects remained large with little regeneration (Joseph and Dyson, 1966b).

More recently, we documented a similar phenomenon in two species of African spiny mice (*Acomys kemp*i and *Acomys percivali*) (Seifert et al., 2012a).

Building on the earlier rabbit studies, we compared 4-mm ear hole regeneration in *Acomys* to ear scarring in outbred Swiss Webster laboratory mice. We observed an accumulation of fibroblast and mononuclear cells at the leading edge of the wounds and an early burst of proliferation in both *Acomys* and *Mus*. As proliferation continued in *Acomys*, regeneration progressed in a proximal distal wave with new hair follicles forming behind the distal proliferative zone. In contrast, proliferation was not maintained in *Mus* and the resulting outgrowth was minimal. Importantly, new hair follicles were not formed in *Mus* and a dense network of collagen fibers formed instead. Approximately 2 weeks postinjury collagen type I was found in greater amounts in the new ECM of *Mus* compared to African spiny mice, where the *Acomys* ECM appeared loosely organized and richer in type III collagen. We qualitatively compared the composition of the ECM during regeneration and saw that fibronectin and tenascin-C were present in the ears of both *Acomys* and *Mus*. Furthermore, we observed increased vasculogenesis and an accumulation of myofibroblasts in the ECM of *Mus*, whereas few myofibroblasts were found in the regenerative ECM of *Acomys*. These studies in rabbit and *Acomys* ears support the notion that aggressive deposition of collagen fibers and accumulation of myofibroblasts in the wound bed antagonize regeneration in favor of scarring. They also point to the temporal control of fibrosis as a potentially important factor regulating a regenerative response. More work is necessary to understand if the local fibroblast to myofibroblast transition is linked with the ECM composition and how new ECM deposition is controlled at the molecular level. Furthermore, it will be vital to further characterize the blastema-like structure that appears during regeneration in order to understand how it forms and the source of cells that ultimately produce the regenerated tissue (Seifert et al., 2012a).

Another situation in which certain species of adult mammals have been shown to regenerate portions of their skin is in large dorsal skin wounds (Billingham and Russell, 1956a,b; Breedis, 1954; Efimov, 1969; Ito et al., 2007; Seifert et al., 2012a). Interestingly, while this has been documented for rabbits, African spiny mice, C57BL/6J, SJL, or a mixture of these strains, it does not occur in rats (Efimov, 1965a), CC57BR, or outbred Swiss Webster mice (Efimov, 1965b; Seifert et al., 2012a). This phenomenon was first documented in a study seeking to understand how contraction affects the wound-healing process (Breedis, 1954). When the edges of a 2-cm wound were prevented from contracting, epidermal placodes were observed first at the wound margins, followed by developing follicles 30 dpi. Dermal papillae developed in association with these regenerating follicles, which ultimately

matured into new hair follicles. Using hematoxylin to stain whole preparations of the tissue throughout the healing process, tracts of cells emanating from existing hair follicles outside the wound bed appeared to contribute cells to the new follicles, although the source of the cells was not determined. The structure of the new dermis was not analyzed.

A subsequent study in rabbits examined the healing process in very large full-thickness excisional wounds ($10\text{--}50\text{ cm}^2$) of various shapes. While they found most wounds contracted almost completely leaving only a thin-line scar, in 14%, contracture halted abruptly after the wounds reached 5 cm^2 or less, and in these animals with incomplete contracture, new hair emerged from the wound epithelium between 40 and 50 dpi (Billingham and Russell, 1956a). Additionally, they found that cortisone treatment or adrenalectomy halted complete contraction and this stimulated hair follicles to regenerate. The regenerated hair was devoid of pigment and similar to observations of regenerating scales, hair orientation was not necessarily in line with that of the surrounding hairs, although the density was not significantly different. Guard hairs were not observed among the regenerated follicles and arrector pilli muscles did not develop either. Examining the dermis, it contained enlarged blood vessels, was approximately twice as thick as the unwounded skin, had a irregular consistency, reduced toughness, and collagen fibers running horizontal to the epidermis suggesting some scarring (Billingham and Russell, 1956a; Efimov, 1969).

The rabbit studies appeared reminiscent of skin regeneration in adult anurans, where epidermis and glands regenerate, but dermis regeneration is not perfect. More recent studies in mice (Gay et al., 2013; Ito et al., 2007) and African spiny mice (Seifert et al., 2012a) have sought to analyze development of regenerating hair follicles and reformation of the dermis. Working with C57BL/6J and SJL strains of laboratory mice, a large wound area was found necessary to stimulate *de novo* regeneration of hair follicles (Ito et al., 2007). Similar to rabbits, these new hairs were not pigmented and older animals required larger wounds (2.25 cm^2) to stimulate regeneration. This phenomenon has been termed wound-induced hair neogenesis (WIHN). A molecular analysis during WIHN showed that molecular markers of hair follicle development were reactivated in the regenerating hair follicles including Krt17, Lef1, *Wnt10b*, and *Shh* in the epithelium and alkaline phosphatase activity in the dermal papilla (Ito et al., 2007). The contribution of follicular bulge cells to interfollicular epidermis and new follicles was assessed. Using a transgenic line that labels predominantly bulge cells, they found that although these cells migrated along tracts into the

wound area, they did not persist or label epidermal cells of the new follicles (Ito et al., 2005). In contrast, using mice that label 70% bulge cells and 50% non-bulge epidermal cells, they found approximately half of the new follicles were labeled indicating a mixed progenitor cell origin as occurs in development. An additional progenitor cell population, and one which likely contributed to this mixed result, is marked by the gene *Lgr6* and found in the central isthmus of the hair follicle (Snippert et al., 2010). Following wound healing, this progenitor cell contributes long term to the basal cells of the wound epidermis, new hair follicles, and sebaceous glands. In order to assess the role of Wnt signaling during follicular regeneration, *Dkk* (a Wnt inhibitor) was expressed prior to and up to 17 dpi (Ito et al., 2007). Inhibiting Wnt signaling up to 10 days after injury had no effect on follicle regeneration, whereas inhibiting Wnt from 11 to 17 days almost completely blocked follicle regeneration supporting a reactivation of signaling pathways involved in hair follicle development. More recently, the same group found a requirement for $\gamma\delta$ T cells during WIHN, specifically as a source of dermal Fgf9 (Gay et al., 2013). Transgenic mice lacking $\gamma\delta$ T cells exhibited >60% fewer new follicles during WIHN and mice lacking *Fgf9* specifically in $\gamma\delta$ T cells produced a similar result (Gay et al., 2013). Unfortunately, neither study examined the quality and extent of dermis regeneration.

More recently, our group reported a similar phenomenon of skin regeneration in large full-thickness wounds of African spiny mice (Seifert et al., 2012a). The skin of these mice is extremely weak (compared to laboratory mouse skin) and easily tears from the body when tension is applied. This unique behavior stimulated our interest in how these animals heal different size wounds. The large size of spiny hairs embedded within the skin made long-term assessment of small wounds impossible because they quickly closed and were practically invisible. Therefore, we used larger 1.5 cm diameter wounds to test the regenerative ability of spiny mice dorsal skin following wounding. Unlike most previous mammalian studies, we focused on both the dermis and epidermis during the repair process. Comparing healing dermis from African spiny mice to outbred Swiss Webster mice, we found that they exhibited a faster rate of reepithelialization and slower deposition of collagen type 1 during new tissue formation. Analysis using picrosirius red suggested the deposition of type III collagen persisted longer and was followed by less aggressive production of type I collagen. We also noted that the dermal architecture appeared more similar to unwounded dermis than to the fibrous scar that persisted in *Mus* wounds. Importantly, we discovered that these mice were capable of regenerating hair follicles

in these wounds beginning approximately 21 dpi. We found that regenerating hair follicles expressed several molecular markers of hair follicle development, similar to those described above in WIHN studies. Building on previous work examining Wnt signaling during hair follicle regeneration (Ito et al., 2007), we found Lef1 protein expression in basal keratinocytes prior to follicle formation which then localized to epidermal placodes, early hair germs and condensing dermal papilla cells. In contrast, we were unable to detect Lef1 protein in *Mus* epidermis and this was coincident with a failure to regenerate hair follicles. Examining the wound bed 4 weeks after injury, we found well-developed hair follicles throughout the wound bed and a regenerated dermis (Seifert et al., 2012a).

Of course, these mammalian studies raise the question of why some mammals have the capacity to regenerate skin while other mammals, including humans, cannot. Future work using the WIHN model should contribute a more complete understanding of the molecular control of hair follicle regeneration and work with nonmodel organisms like the African spiny mouse will help determine if similar or different mechanisms underlie skin regeneration. More importantly, in the context of skin regeneration in vertebrates, not just mammals, the data strongly suggest that the two main compartments of skin, epidermis, and dermis, have regeneration capacities that are independent of each other, although they still appear to require an interaction for complete regeneration of skin appendages to occur.



7. CONCLUDING REMARKS

Although wound-healing research over the last century has contributed to a deep understanding of human scarring, regenerative failure of the skin is clearly evident in human wounds. Comparisons between adult wound repair where a scar occurs and healing of fetal skin at early gestational stages when it undergoes scar-free wound healing have borne out clear cellular and molecular differences. However, none of these differences have allowed the induction of scar-free healing and good dermal regeneration in adult mammalian skin wounds. We suggest this may be because the comparison between the fetus and the adult is likely to be misleading. The fetal tissue is in an immature state and may simply respond to damage by continuing with its developmental program. In contrast, the fully differentiated adult tissue must respond to injury and restore the physiological changes that result both locally and systemically. Terminally differentiated cells or local progenitors must dedifferentiate, reenter the cell cycle, and activate genetic

programs normally deployed during embryonic development. Given the undifferentiated state of fetal tissue, these studies will not reveal how dedifferentiation can occur. We propose that a more appropriate comparison to understand how skin injury can be coaxed to regenerate is between adult vertebrate animals that can naturally regenerate skin and adult mammalian models of wound healing. We have reviewed these cases in fishes and amphibians and have also highlighted less commonly used mammalian models of wound healing and regeneration including rabbits and African spiny mice.

While these nonhuman systems possess positive and negative aspects as study systems, the fact remains that as adults many can regenerate skin scar-free, and thus are extremely useful in their ability to provide insight into mechanisms that regulate skin regeneration. Researchers must put aside their inherent system biases and look to synthesize data from multiple animal models. Mammalian wound-healing researchers should welcome new ideas to the field and researchers using nonmammalian systems should look to synthesize findings across different animal models. Unfortunately, however, this narrow model-centric thinking continues ([Richardson et al., 2013](#)) and typifies a hurdle present in many fields.

Nonetheless, when wound healing is examined across all of these models, several important factors emerge ([Fig. 4.5](#)). First, there is little evidence to suggest that the rate of reepithelialization plays a role in the ability to regenerate the skin. Animals that are capable of skin regeneration can take weeks to reepithelialize the wound bed. While some regenerating species exhibit very fast reepithelialization times, this appears related to whether the epidermis is mucogenic or keratinized. Second, regeneration of the dermis occurs after formation of a fibrous granulation tissue in the wound bed. This fibrous tissue is subsequently remodeled rather than the regenerated dermis forming *de novo* as it is during development. Third, the mature immune system is not inhibitory to regeneration and it is much more likely that the cytokine profile of inflammatory cells, rather than the cells themselves, is the crucial factor directing the early fibrotic response. Indeed recent studies in the mouse have revealed how a single chemokine delivered by one particular immune cell type can set up a feedback loop leading to the induction of hair follicle regeneration ([Gay et al., 2013](#)). How growth factors direct dermal regeneration is completely unknown, but a similar feedback loop may exist between local fibroblasts and components of the ECM. With an ever expanding molecular toolkit, the future is bright for studies using comparative approaches to understand the basic mechanisms underlying skin regeneration.

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