

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 regenera-

tion, 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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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'-TGGGTTTTGATGTGTTTGCT-3' and Ribosomal protein L19 forward, CATGGGCACTGGTAAGAGAAAAG; 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

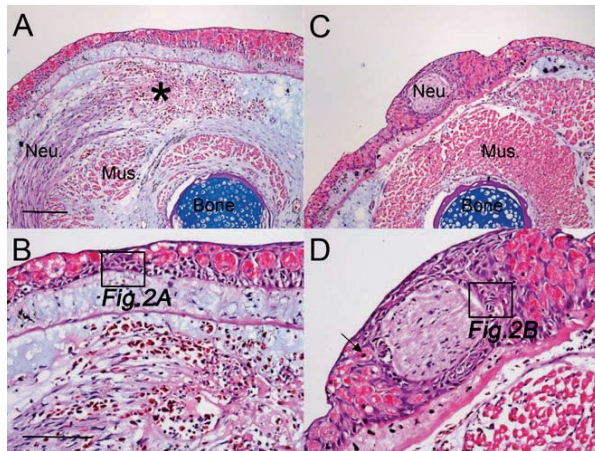


Fig. 1. Nerve deviation under or on the dermal collagen layer. (**A**, **B**) A nerve was deviated under the collagen layer. The nerve ends were seen under the dermal collagen layer (**A**, asterisk). Panel **B** is a higher magnification of panel **A**. (**C**, **D**) A nerve was deviated on the dermal 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 μ m. Scale bar in **C** = 100 μ m. (**A**) and (**C**) are the same magnification as (**B**) and (**D**). Arrow in (**D**) indicates a Leydig cell in the epidermis.

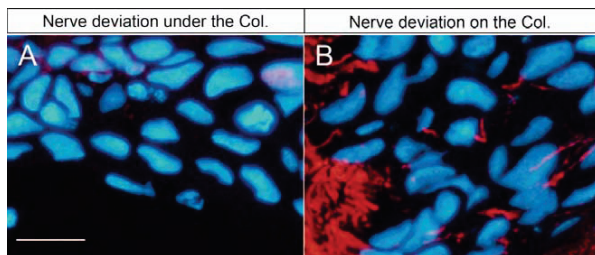


Fig. 2. Visualization of axon fibers. Axon fibers were stained with anti-acetylated tubulin antibody (red) and nuclei with DAPI (blue). (**A**) The deviated nerve under the dermal collagen layer did not reach the axons in the overlying epidermis. (**B**) Axon fibers could be seen in the epidermis. The 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 μ m. **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 sec-

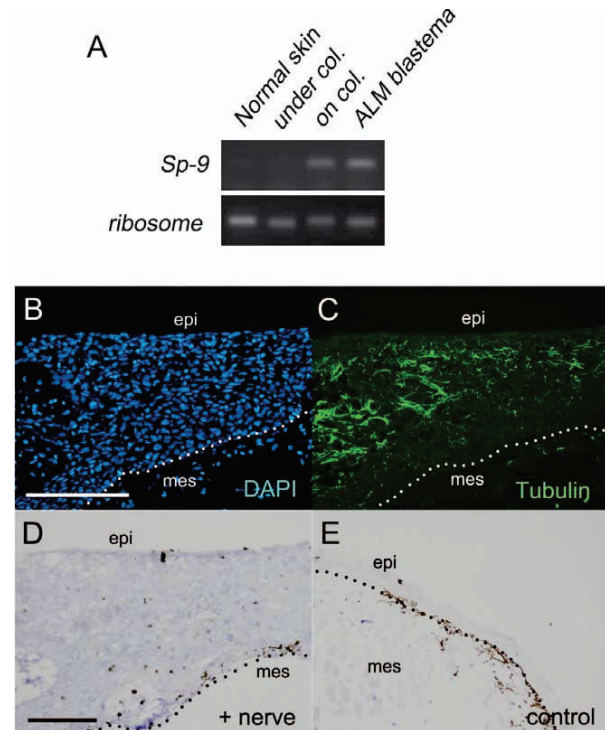


Fig. 3. *Sp-9* expression by nerve deviation. (**A**) RT-PCT analysis. *Sp-9* was not detected in normal skin. The rerouted nerve under the collagen layer (lane 2) did not activate *Sp-9* expression. In contrast, the deviated nerve on the collagen layer (lane 3) increased *Sp-9* expression. The ALM blastema 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 μ m. Scale bar in **D** = 100 μ m. (**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).

tion 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.

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. 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

(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.

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

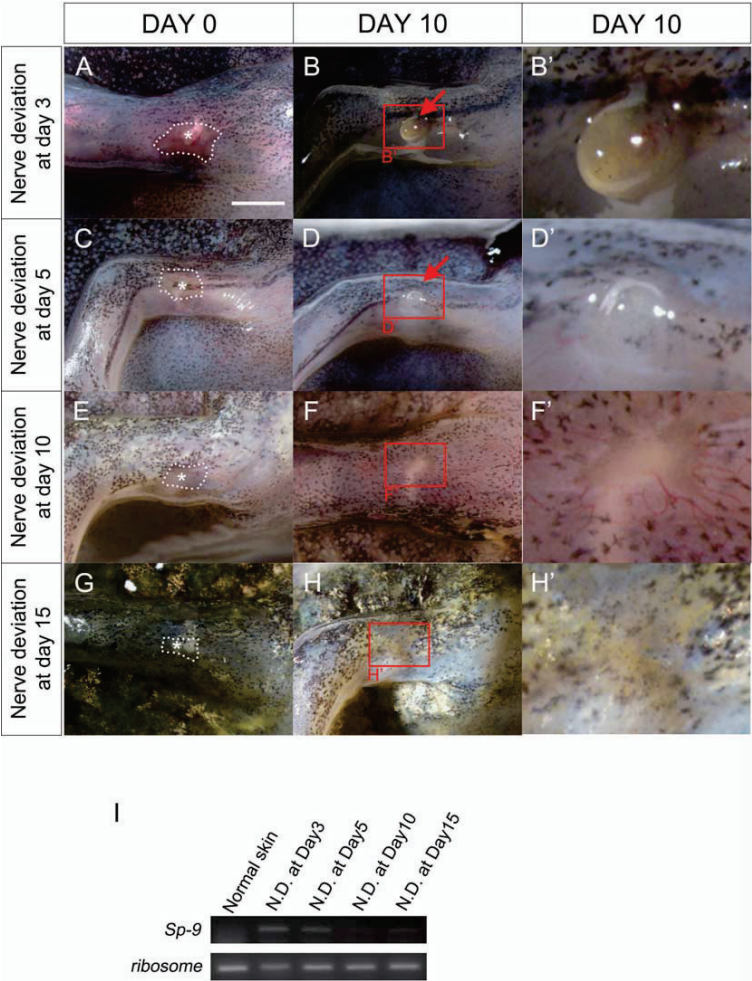


Fig. 4. Nerve deviation to wounds on specific days after wounding. (A–H) Anterior view of the limb with the rerouted nerve immediately (A, C, E, G) and 10 days (B, D, F, H) after surgery. Wound margin and putative wound margin were outlined by the dotted line in (A), (C), (E), and (G). Asterisks indicate 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.

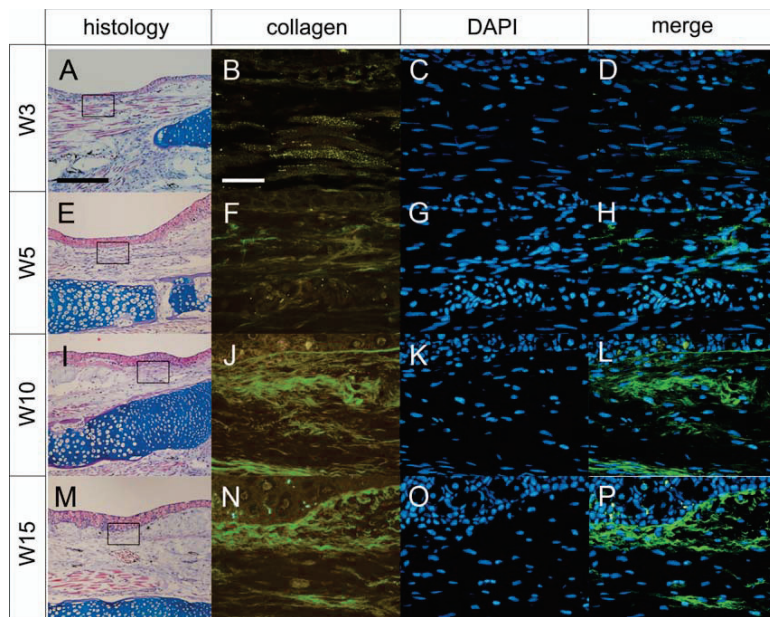
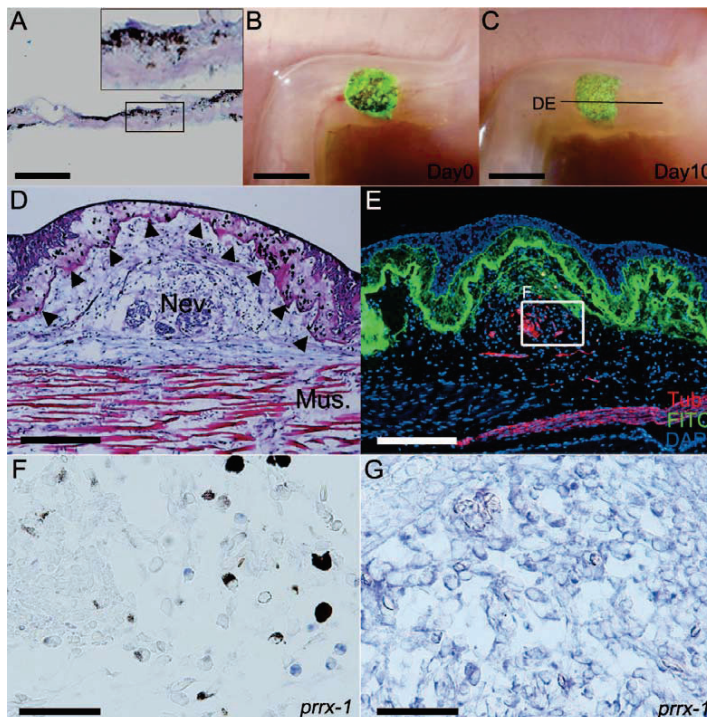


Fig. 5. Collagen reorganization during wound healing. (A–D) Three-day-old wound. (E–H) Five-day-old wound. (I–L) Ten-day-old wound. (M–P) Fifteen-day-old wound. (A, E, I, M) Alcian blue and hematoxylin and eosin staining. (B, F, J, N) Collagen was visualized by immunohistochemistry (green). (C, G, K, 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 μ m. (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 μ m, and 100 μ m, respectively. E and G are the same magnification as D and F, respectively.

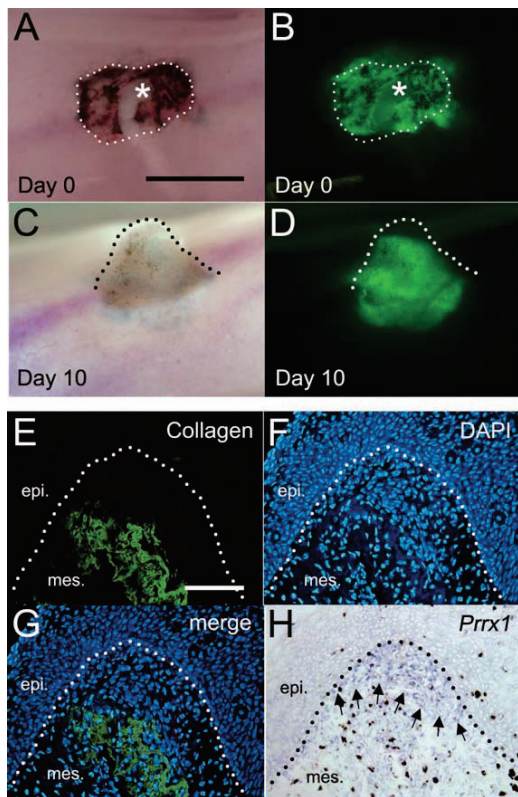


Fig. 7. Collagen sheet graft below the rerouted nerve did not inhibit blastema induction. Anterior view of the limb with the rerouted nerve immediately (**A, B**) and 10 days (**C, D**) after surgery. Asterisks in (**A**) and (**B**) indicate the end of the rerouted nerve. The grafted collagen was stained 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 μ m, 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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