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Neurotrophic regulation of fibroblast dedifferentiation during limb skeletal regeneration in the axolotl (*Ambystoma mexicanum*)

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ABSTRACT

The ability of animals to repair tissue damage is widespread and impressive. Among tissues, the repair and remodeling of bone occurs during growth and in response to injury; however, loss of bone above a threshold amount is not regenerated, resulting in a “critical-size defect” (CSD). The development of therapies to replace or regenerate a CSD is a major focus of research in regenerative medicine and tissue engineering. Adult urodeles (salamanders) are unique in their ability to regenerate complex tissues perfectly, yet like mammals do not regenerate a CSD. We report on an experimental model for the regeneration of a CSD in the axolotl (the Excisional Regeneration Model) that allows for the identification of signals to induce fibroblast dedifferentiation and skeletal regeneration. This regenerative response is mediated in part by BMP signaling, as is the case in mammals; however, a complete regenerative response requires the induction of a population of undifferentiated, regeneration-competent cells. These cells can be induced by signaling from limb amputation to generate blastema cells that can be grafted to the wound, as well as by signaling from a nerve and a wound epithelium to induce blastema cells from fibroblasts within the wound environment.

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Introduction

The ability of animals to repair tissue damage in response to injury is widespread and impressive. In some instances, this ability extends to regenerating most of the entire body (e.g. planaria); however, for most vertebrates, including humans, regeneration is restricted to the level of tissues such as bone, muscle, nerves and blood vessels. In spite of the ability to regenerate individual tissues, regeneration of organs that integrate the structures and functions of the individual tissues does not occur. A notable exception is urodele amphibians (salamanders), which are unique among adult vertebrates in their ability to regenerate complex organs, including limbs. Among the tissues and organs that can regenerate in salamanders, the limb has been most extensively studied, allowing for much of our current understanding of the mechanisms regulating organ regeneration in adult tetrapods. The challenge of regenerative medicine is to discover how to integrate and orchestrate the regenerative responses of the various component tissues in order to regenerate functionally and structurally complex organs (Bryant et al., 2002; Endo et al., 2004).

The skeleton is one of the tissues that can regenerate in humans and other tetrapods. Bone and cartilage are dynamic tissues that are constantly being remodeled during growth in youth, as well as in old age. In addition, injuries to the skeleton typically heal well and small structural deficiencies are replaced. The endogenous regenerative response of mammalian bones is well understood and initially involves formation of a soft callus (fibrocartilage) derived from mesenchymal progenitors (Schindeler et al., 2008). This structure provides mechanical support to the fracture, and is the template for the eventual formation of woven bone that restores structure strength to the regenerated bone. This intrinsic regenerative response can heal the end of apposed bones as well as fill in small gaps between the ends of the broken bones. In the later case, this gap can be progressively widened with progressive callus formation, which is the basis of distraction osteogenesis therapies to elongate skeletal elements.

In spite of this intrinsic regenerative ability, loss of bone above a threshold level, a “critical-size defect” (CSD) is not regenerated (Schmitz and Hollinger, 1986). The challenge to regenerate a CSD is a major focus of therapies in regenerative medicine. Most of these therapies are designed to orchestrate the interaction of grafted cells with the potential to regenerate the skeleton, with scaffolds that bridge the CSD. In the end the goal is to provide a new skeletal element that integrates into and has the structural properties of the endogenous skeleton (Schmidmaier et al., 2008). In spite of progress in achieving these goals, long bone defects remain an unsolved and challenging clinical problem.

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As in humans and other vertebrates, salamanders also fail to regenerate a CSD, in spite of their ability to regenerate an entire amputated limb (Goss, 1969; Hutchison et al., 2007). A salamander limb is composed of the same tissues as a human limb, and each regenerates by a comparable mechanism involving the growth from existing tissues in the stump (nerves and blood vessels) or from adult stem cells, such as muscle satellite cells (Gardiner, 2005; Kragl et al., 2009; Morrison et al., 2006). Nevertheless, as in mammals, a mid-diaphyseal radial deletion is not regenerated (Hutchison et al., 2007). Unlike mammals, the salamander arm does have the potential to regenerate the missing skeletal element. If the ulna is removed surgically and the limb is amputated, the ulna is regenerated distal to the amputation plane even though it was absent in the proximal stump at the time of amputation (Goss, 1969). Of relevance to our study is the observation that although the distal ulna is regenerated, the proximal defect is not (Goss, 1969). Therefore the limb stump cells have the ability to regenerate an excised ulna (equivalent to a CSD), and do so in response to signals that induce dedifferentiation and blastema formation (Bryant et al., 2002), but fail to do so in the absence of those signals.

These classic studies (Goss, 1969) are a reminder that a lack of regeneration (such as a CSD) is not necessarily a consequence of a lack of regenerative ability, but may result from a failure to progress through the early, requisite steps of regeneration (Muller et al., 1999). Collectively these early steps result in blastema formation via a developmental process referred to as “dedifferentiation.” In spite of the essential role of dedifferentiation in salamander limb regeneration, little is known about this phenomenon at the level of cellular and molecular mechanisms. The current operational definition of dedifferentiation is that it is the process by which a blastema is formed from progenitor cells present in the uninjured limb, and involves the reacquisition of embryonic-like developmental potential as indicated by the re-expression of embryonic genes (Han et al., 2005; Satoh et al., 2008b). By this view, cells in the limb stump revert to an embryonic state (limb bud), and regeneration progress as a recapitulation of limb development. Thus, dedifferentiation is the early phase of regeneration leading to formation of blastema cells that function like limb bud cells to re-develop the lost limb structures.

Although progenitor cells for all the limb tissues eventually contribute to the blastema, the early blastema is derived from cells within the connective tissues of the injured limb (Bryant et al., 2002; Kragl et al., 2009; Muneoka et al., 1986). Cells from other tissues are lineage restricted (e.g. myoprogenitor cells derived from muscle-associated satellite cells) and migrate into the blastema after it forms and begins to grow (Bryant et al., 2002; Kragl et al., 2009). Cells from the connective tissue (fibroblasts) reacquire the developmental potential their progenitors had in the embryo, and give rise to the connective tissue-related tissues of the regenerated limb; including cartilage, bone, ligaments, tendons and loose connective tissue (Kragl et al., 2009; Lheureux, 1983; Muneoka et al., 1986). In addition, these cells within the connective tissue of the limb are the source of the signals that regulate growth and pattern formation during regeneration (Bryant et al., 2002; Endo et al., 2004; Gardiner et al., 1986; Muneoka and Bryant, 1984; Rollman-Dinsmore and Bryant, 1982). Therefore, the challenge to inducing regeneration of skeletal defects lies in understanding the mechanisms regulating the state of differentiation and developmental potential of connective tissue fibroblasts.

The phenomenology of fibroblast dedifferentiation and blastema formation are well understood, even though the molecular details of the underlying signaling pathways are not. The importance of a specialized regeneration epithelium has long been recognized (Tassava and Garling, 1979; Thornton, 1957; Thornton, 1960), and its formation and function is induced and dependent upon signaling from the nerve (Satoh et al., 2008b). Nerve signaling appears to be required at additional steps in the regeneration cascade. The loss of

nerve signaling (denervation) or the loss of a functional regeneration epithelium (e.g. surgical removal or the grafting of mature skin to inhibit its formation) results in the same phenotype, regenerative failure. Although most studies have focused on dermal fibroblasts as the target cells of nerve/RE signaling, fibroblasts in the connective tissues surrounding the skeletal elements appear to function the same as dermal fibroblasts during regeneration (Gardiner and Bryant, 1989; Muneoka et al., 1986). Given the crucial role of the signaling from nerves and the regeneration epithelium in the induction of dermal fibroblast dedifferentiation and blastema formation, we hypothesize that the same pathways will induce fibroblasts within the center of the limb to dedifferentiate and give rise to multipotential cells that could regenerate an excised skeletal element.

In this paper, we report on a novel model for the regeneration of a critical-size defect (the Excisional Regeneration Model) that can be utilized as an assay to identify signals to induce fibroblast dedifferentiation and skeletal regeneration in salamanders, and potentially in humans. Although this regenerative response is in part mediated by BMP signaling, as is the cases in mammals, a complete regenerative response requires the induction of an undifferentiated, regeneration-competent population of cells. These cells can be generated exogenously by limb amputation leading to blastema cell formation, and can also be generated endogenously in response to signals from a deviated nerve in association with a wound epithelium. The host environment created by excision of the skeletal element is both permissive and instructive for these regeneration-competent cells to replace the missing limb segment.

Materials and methods

Animals and surgical procedures

Experiments were performed on axolotls (*Ambystoma mexicanum*) measuring 8–12 cm from snout to tail tip that were spawned at the University of California, Irvine or the *Ambystoma* Genetic Stock Center at the University of Kentucky. For all surgeries, we anesthetized animals in a 0.1% solution of MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma), pH 7.0. Animals were kept anesthetized and covered with moist lab tissues for 1 h post-surgery.

To create a mid-diaphyseal radial defect, we made three incisions in the skin overlying the anterior quarter of the lower arm so as to create a skin flap that was still attached to the arm skin on the forth side of the square. We reflected the flap back to expose the underlying soft tissues, and then reflected the muscle fibers to expose the radius. We then used microforceps and iridectomy scissors to dissect the adherent connective tissues, blood vessels and nerves free from the radius and removed a 2-mm segment from the mid-diaphyseal region. We then repositioned the soft tissues and the skin flap, which healed into place without sutures by reepithelialization within 6–8 h (Carlson et al., 1998; Satoh et al., 2008b). To create defects in which a wound epithelium was formed, four incisions were made initially to remove the skin square, and the underlying muscles were removed to expose the radius. After creating the radial defect, we allowed the wound to reepithelialize, which occurs rapidly in these animals, and was complete within 6–8 h (Carlson et al., 1998; Satoh et al., 2008b).

To deviate a nerve to the radial defect, we surgically exposed the pair of nerves that are located between the radius and ulna. We severed these nerves distally and rerouted them to the cavity created by excising the radius. In those experiments in which skin and muscle were removed to induce formation of a wound epithelium, the nerves were positioned such that they eventually were located immediately beneath the wound epithelium after it formed. To test for the requirement for innervation, we denervated limbs by surgically exposing and transecting the third, fourth and fifth brachial nerves distal to the brachial plexus at the scapula level.

Grafting of cells, BMP2 beads, and Noggin beads

Cells for grafting into the host site created by excision of the radius were obtained from either medium/late bud blastemas (blastema cells), the dermis of uninjured limb skin (dermal fibroblasts), or articular cartilage from the epiphyses of the radius, ulna and humerus (chondrocytes). Blastemas were induced by amputating limbs at either proximal (mid-humerus) or distal (mid-radius/ulna) levels. The amputated bone was trimmed so as not to protrude from the amputation surface. Blastemas at the medium/late bud stage were collected, the epithelium was removed manually with microforceps, and the underlying mesenchymal portion was dissociated enzymatically. To obtain dermal cells, we removed the full-thickness skin from an arm that we removed by a proximal limb amputation, removed the keratinocytes with Ca/Mg-free EDTA, and dissociated the dermal tissue enzymatically. To obtain chondrocytes, we dissected out the epiphyses of the limb bones from an arm that was removed by a proximal limb amputation and dissociated the tissue enzymatically. Tissues were dissociated using 0.25% trypsin/PBS. The suspension of dissociated cells was neutralized with 10% FBS-containing medium and then washed with serum-free DMEM.

Cells were grafted after we encapsulated them in a collagen clot. An acidic collagen solution was neutralized by sodium bicarbonate on ice at a final collagen concentration of 36 mg/ml. The neutralized collagen solution was mixed with the cells (approximately 5×10^6 cells/ml). The collagen clot formed after several minutes at room temperature, after which we grafted a clot containing cells with a volume of approximately 10 μ l. Gelatin microspheres were prepared according to the method described previously (Tabata et al., 1999). We selected 300 μ m to 500 μ m beads for grafting. Beads were implanted into host sites created by excision of the radius in order to test for the effects of BMP2 and Noggin (R&D systems). Experimental beads were soaked in a solution of 1 μ g/ μ l BMP2 protein or Noggin protein, and control beads were soaked in BSA (0.1%).

Cloning of axolotl *Bmp2*

We used RT-PCR with degenerate primers (forward GCCAACA-CYGTGMKSAGYTTCC and reverse CCCTCCACNACCATSTCCTG) to isolate a partial clone for the axolotl ortholog of *Bmp-2*. Total RNA was prepared using the RNeasy Kit (Qiagen) from stages of limb regeneration (early bud to palette stage blastemas). We used (dT) 12–16 as a primer to reverse transcribe cDNA from total RNA (500 ng) with Superscript III (Invitrogen) according to the manufacturer's protocol. The reaction mixture was diluted three-fold with TE, and used as template for PCR. The PCR reactions were performed using rTaq (Takara) with the following conditions: 98 °C for 2 min; followed by 32 cycles of 98 °C for 15 s, 50–62 °C for 15–45 s, 72 °C for 1 min; and 72 °C for 2 min. The PCR reaction products were purified by gel electrophoresis, cloned into the TOPO pCR2 cloning vector (Invitrogen) using the manufacturer's protocol, and sequenced by MCLAB (Molecular Cloning Laboratories, San Francisco). The full-length sequence for axolotl *Bmp-2* recently has been reported (GenBank EU339232), and we obtained an 856-bp fragment from the open-reading frame corresponding to nt987–nt1843.

Probe synthesis, *in situ* hybridization and immunohistochemistry

For probe synthesis, the *Bmp-2* template was amplified by PCR with exTaq (Takara) using primers to M13 sequence flanking the cloned axolotl sequences in the TOPO pCR2 vectors. PCR fragments were purified and used as a probe template. Probe synthesis was performed with Sp6 RNA polymerase for 4 h. Probes for *Prrx-1*, *Sp9*, and *Msx-2* were synthesized as described previously (Satoh et al., 2007, 2008b). We note that the name for the pair-related homeobox 1 gene has been standardized in the NCBI databases (*Prrx-1*), whereas

we had previously referred to the axolotl ortholog as *Prx-1* (Satoh et al., 2007). Procedures for *in situ* hybridization to tissue sections were based on those previously published in detail (Satoh et al., 2007).

Immunohistochemistry was performed as described previously (Satoh et al., 2007) using anti-type II collagen (Developmental Studies Hybridoma Bank, II-II6B3, 1:200), anti-GFP (Molecular Probes, 1:200), anti-BrdU (Roche, 1/200), anti-mouse IgG-alkaline phosphatase (Invitrogen), anti-mouse IgG-Alexa 488 (Invitrogen), anti-mouse IgG-Alexa 594 (Invitrogen) and anti-rabbit IgG-Alexa 488 (Invitrogen). Nuclei were stained with DAPI.

Histology

To visualize cartilage in the regenerated skeletal elements in whole-mount preparations, we fixed samples in 10% formalin diluted with 40% Holtfreter's solution for 3–4 h. We then incubated the samples in 29% formalin/70% ethanol/1% HCl (Solution A) for 3–4 h, after which they were stained with 0.1% Alcian Blue in Solution A overnight at 42 °C. Samples were washed with 2% KOH without agitation for 2 h at room temperature, followed by a solution of 2% KOH/50% Glycerol for 1–3 days (depending on the size of the tissue sample). Samples were cleared in 100% glycerol prior to photography. To visualize regenerated cartilage in tissue sections, slides were hydrated and stained with a 0.01% Alcian blue/0.1% HCl solution for 30 min, followed by standard hematoxylin and eosin staining.

Results

Axolotls did not regenerate a critical-size defect

Deletions of a 2-mm portion of the radius or tibia in an axolotl persisted for at least 20 days after surgery (Fig. 1, Table 1). The anatomy of tetrapod limbs is conserved, and the skeleton of an axolotl is very similar to a human (Figs. 1A, B), with the obvious difference that an axolotl arm has only four phalanges (Fig. 1A). When a mid-diaphyseal defect was created, the skeleton appeared to resorb back from the cut edge over the next several days (Figs. 1E, F). Within 5 days post-injury, the cartilage matrix was noticeably deficient within a zone extending about 500 μ m from the cut edge of the bone, and by 20 days neither the matrix nor the chondrocytes were evident in this region (Fig. 1G). In contrast to the cartilage within the bone, the peripheral portion of the skeletal element (including the perichondrium) was not resorbed. Over this same time period, newly synthesized cartilage was observed at the cut end of the bone, where it formed a soft callus (Figs. 1G, H). The callus extended on the order of 50–150 μ m laterally and distally to the cut end of the bone. The gap that persisted (essentially the entire 2 mm defect) was filled with fibrous tissue that we presume to be an extension of the loose connective tissue of the limb stump. Only 1 of 14 limbs exhibited a regenerative response in which the gap was observed to be partially filled in with a cartilaginous outgrowth (Table 1). An additional limb in which the ulna was injured during surgery and the limb became bent such that the ends of the deleted radius became apposed and grew together was not included in the data reported in Table 1. These findings are consistent with a previous report that a larger defect (4 mm) was not regenerated after a period of 7 months post-injury (Hutchison et al., 2007). Thus a 2-mm excisional wound in an axolotl (8–12 cm snout to tail tip) is a critical-size defect.

Callus formation did not appear to involve blastema formation

The localized regenerative response leading to callus formation appeared to involve the recruitment and proliferation of chondrogenic cells of the perichondrium and loose connective tissues surrounding the radius (Figs. 1G, H). Proliferative cells (incorporating BrdU) were observed among the cells associated with the cut ends of

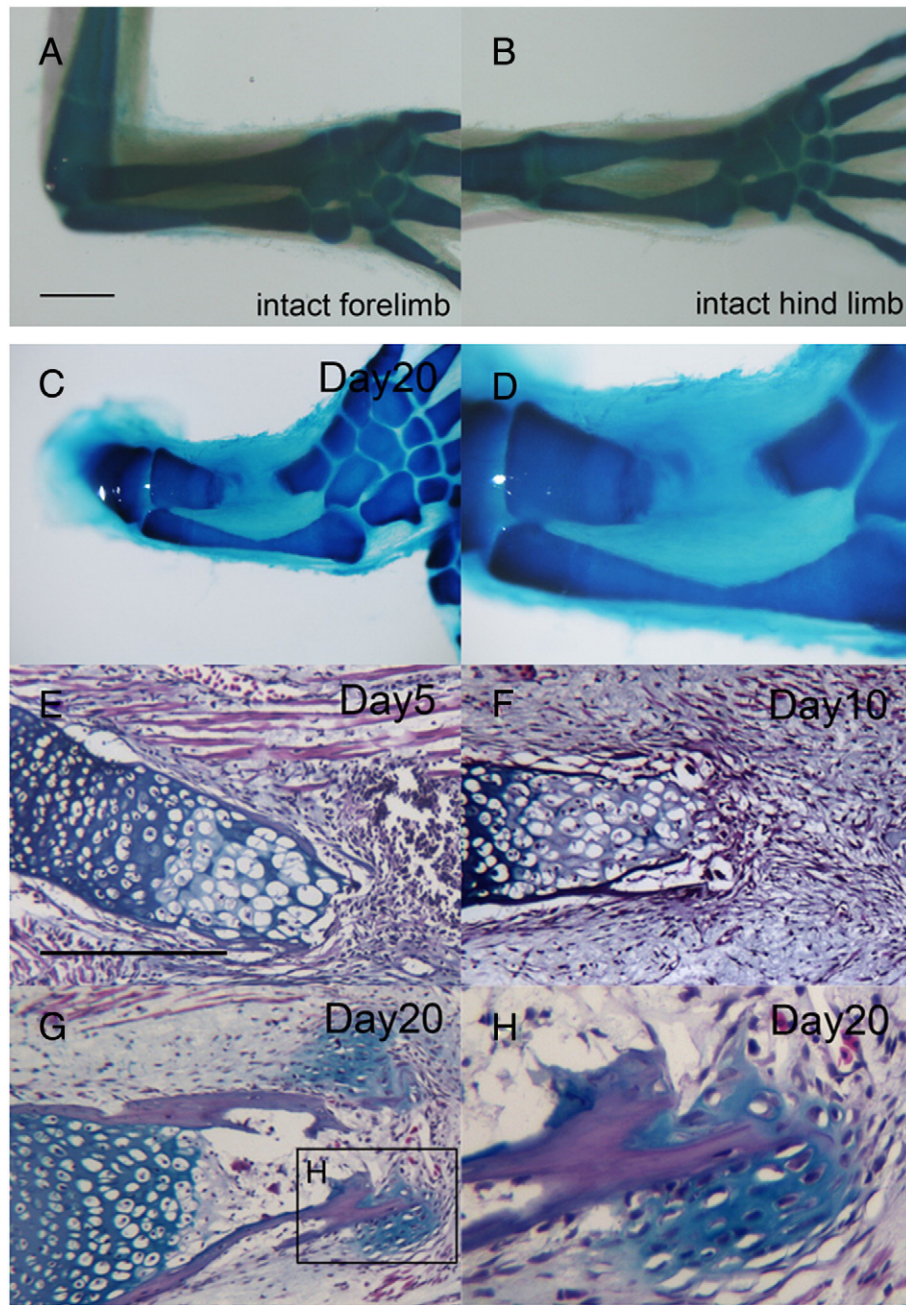


Fig. 1. Callus formation in association with a critical-size defect in the axolotl zeugopod. Normal skeletal anatomy of an axolotl forelimb (A) and hind limb (B) was visualized by Alcian blue staining. Excision of the middle third of the radius (C) or tibia (not illustrated) resulted in the persistence of a critical-size defect 20 days post-surgery. The forelimb in C is illustrated at higher magnification in D. The matrix surrounding the cells at the cut edge of the excision was decreased in association with resorption of the end of the radius at day 5 post-surgery (F). The cut end of the radius was beginning to expand by day 10 post-surgery (F), and a cartilaginous, Alcian blue-stained callus was well formed by day 20 post-surgery (G). The area within the box in G is illustrated at higher magnification in H. The limbs are oriented such that distal is toward the right and anterior is toward the top of each image. Scale bar in A is 2 mm (B–C are at the same magnification) and in E is 0.5 mm (F, G are at the same magnification).

the radius 10 days after surgery (Figs. 2A, A'). At day 20 post-surgery, when the callus was well developed, proliferative cells were localized to the region corresponding to the callus (Figs. 2B, B').

The regenerative response associated with an excised radius appeared to be different from regeneration of an amputated limb. In response to amputation, cells of the stump connective tissue dedifferentiate and migrate to the center of the wound where they form blastema cells that proliferate and give rise to the new connective tissues of the limb, include the skeletal tissues (Bryant et al., 2002; Gardiner et al., 1986; Muneoka et al., 1986). Callus formation appeared to result from a more localized proliferation and contribution of cells directly associated with the cut ends of the

excised skeletal element. To test whether callus formation involved blastema cell formation, we analyzed the expression of two genes that are markers for undifferentiated blastema cells, *Prrx-1* and *Msx-2* (Satoh et al., 2007). Although *Msx-2* was expressed at high levels in regenerating calluses (Figs. 3A, A'), *Prrx-1* was expressed at a low level in only a few cells associated with the callus (Figs. 3B, B'). Thus it appears that most of the cells involved in callus formation are not equivalent to blastema cells (Satoh et al., 2007).

Although dedifferentiation and blastema cell formation were not induced, the wound environment created by excision of the radius was permissive for the survival and maintenance of blastema cells once they were formed. *Prrx-1* expressing cells from a medium bud

Table 1
Regeneration of radial critical-size defects in the axolotl (*Ambystoma mexicanum*).

Experiment	Complete	Partial	Defect	Total
Excision (no graft)	0 (0%)	1 (7%)	13 (93%)	14
BMP2 bead graft	9 (75%)	1 (8%)	2 (17%)	12
Blastema cell graft	7 (64%)	2 (18%)	2 (18%)	11
Fibroblast graft	0 (0%)	2 (20%)	8 (80%)	10
Chondrocyte graft	2 (33%)	4 (67%)	0 (0%)	6
Deep wound + nerve deviation	4 (44%)	4 (44%)	1 (12%)	9
Deep wound + nerve deviation + fibroblast graft	2 (33%)	2 (33%)	2 (33%)	6
Deep wound (no nerve deviation)	0 (0%)	2 (40)	3 (60%)	5
Deep wound + nerve deviation + denervation	0 (0%)	0 (0%)	3 (0%)	3
Excision + nerve deviation	0 (0%)	1 (12%)	7 (88%)	8
Excision + denervation	0 (0%)	0 (0%)	4 (100%)	4

blastema of an amputated limb continued to express high level of *Prrx-1* after being grafted into the site of the excised radius (Figs. 3C, C'). Thus the wound environment provided signals that were permissive for blastema cell maintenance, even though the signals to induce dedifferentiation and localized blastema cell formation were not present.

Callus formation by perichondrocytes was associated with endogenous BMP signaling

BMP signaling is among the most important regulatory molecules in bone formation and healing (Reddi, 1998, 2005). To determine if BMP signaling was activated in response to excision of the radius, we

cloned an 859-bp fragment from the open-reading frame of the axolotl *Bmp-2* ortholog. Expression of *Bmp-2* was locally upregulated in chondrocytes and loose connective fibroblasts in association with the cut ends of the radius (Fig. 4A). *Bmp-2* expression was not detected in uninjured regions of the radius just a few millimeters more proximally or distally in association with the epiphyses of the radius (Fig. 4B). In addition, inhibition of endogenous BMP signaling inhibited callus formation (Fig. 5). We implanted beads soaked in the BMP antagonist Noggin into the radial defect, and observed a decrease in the extent of callus formation at day 20 post-surgery (compare Figs. 5G, H with Figs. 1G, H). We presume that callus formation was not completely inhibited because only a limited amount of Noggin protein could be delivered to the wound site, and it was released and was dispersed over a relatively short time period. Assuming that endogenous BMP signaling occurred over a longer time period, callus formation would then be reinitiated. Taken together, these results indicate that BMP signaling was induced in response to excision of the radius, and it functioned to mediate an endogenous regenerative response leading to callus formation.

Given that endogenous BMP signaling functioned during radius regeneration, we tested whether this regenerative response could be enhanced by the delivery of exogenous BMP protein. We increased the level of BMP signaling by implanting beads soaked in BMP2 into the radial defect, and observed an enhanced chondrogenic response, leading to closure of the defect (Figs. 5A, B, E, F) in most of the cases (Table 1, $n = 9/12$). The outgrowth of cartilage was continuous with the cut ends of the radius (Figs. 5E, F) and extended asymmetrically along the axis of the radius toward the cut end on the opposite side of the defect. This outgrowth response was strikingly different from the

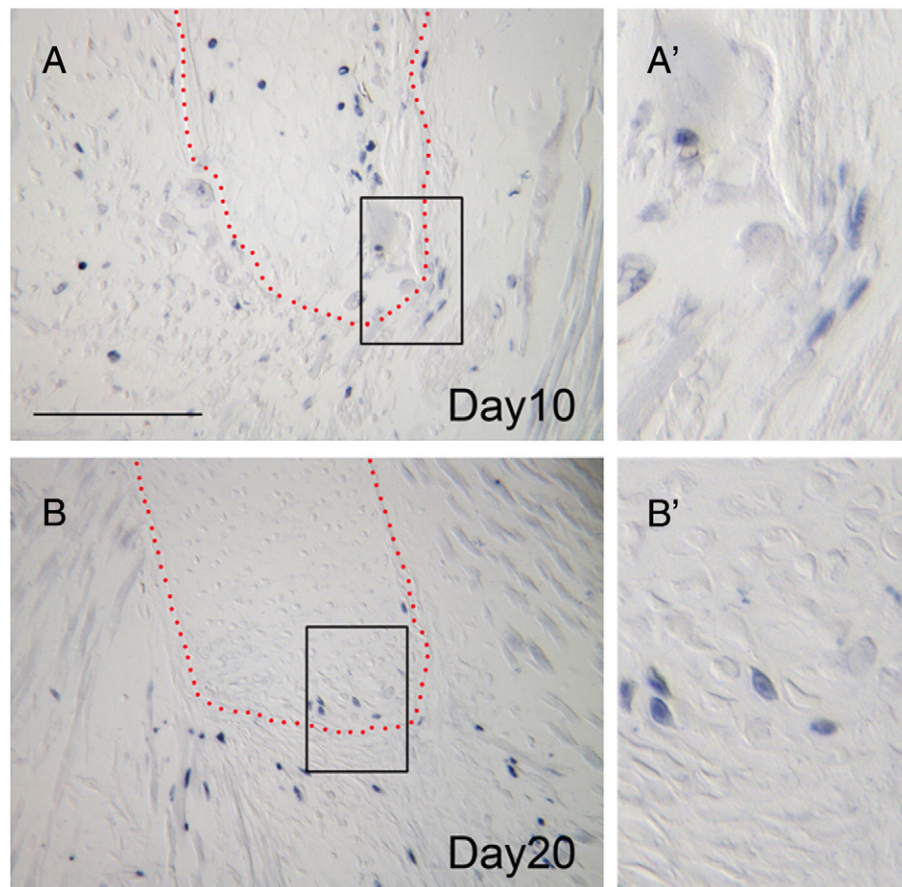


Fig. 2. Cell proliferation in association with the cut ends of the radius. Cells that had incorporated BrdU were observed around the radius and scattered throughout the connective tissue at day 10 post-surgery (A, A'). By day 20 post-surgery, BrdU positive cells were localized to the end of the amputated radius in association with the regenerated callus (B, B'). The dotted red lines in A and B indicate the boundary between the radius and the surrounding soft tissues. The boxed areas in (A) and (B) are illustrated at higher magnification in A' and B' respectively. Scale bar in A is 0.3 mm (B is at the same magnification).

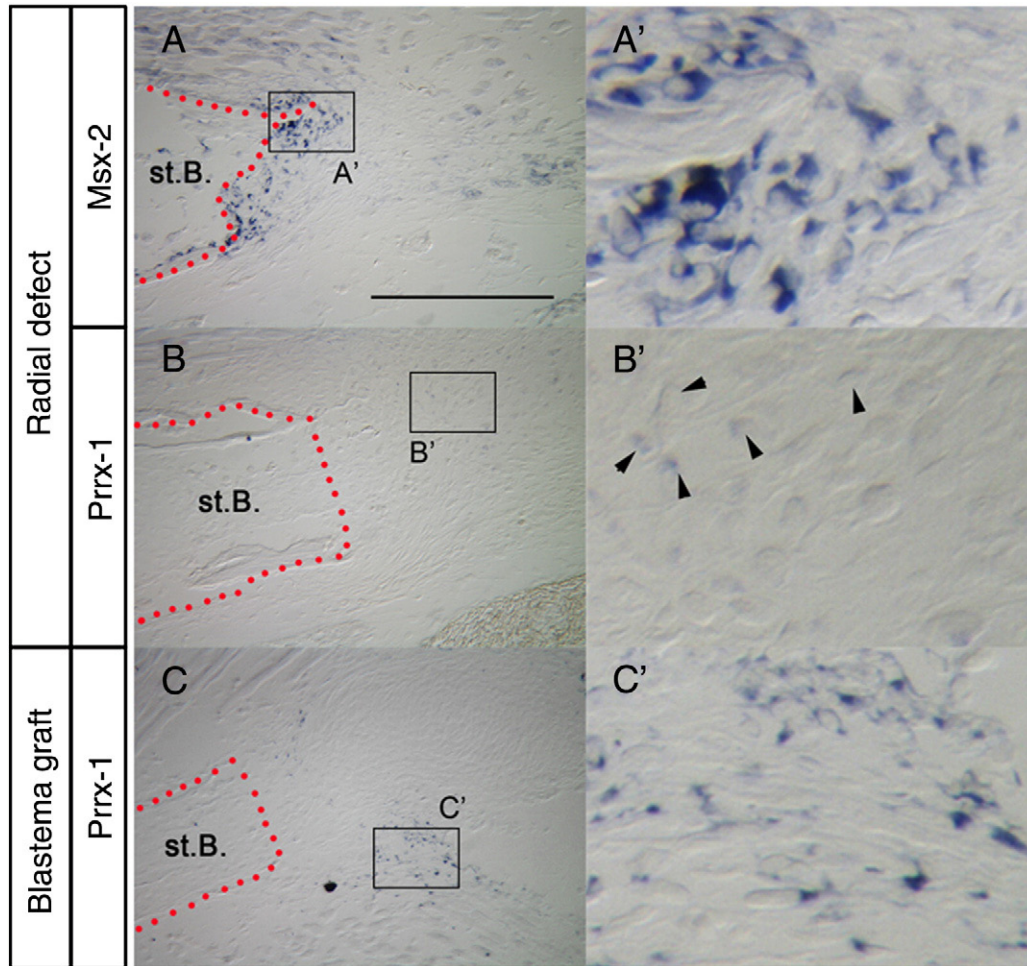


Fig. 3. Expression of *Prrx-1* and *Msx-2* in the callus formed in a radial defect, and in blastema cells grafted into a radial defect. *Msx-2* was expressed at high levels in cells associated with the callus that formed 20 days post-surgery (A, A'), but *Prrx-1* was only expressed at very low levels (B, B'). Expression of *Prrx-1* was high in blastema cells (Satoh et al., 2007) and remained high 20 days after they were grafted into a radial defect (C, C'). Most of the grafted blastema cells had already differentiated into chondrocytes (see Fig. 7); however, undifferentiated blastema cells expressing *Prrx-1* were still present. Dotted red line indicates the border of the stump bone (st.B). The boxed areas in A–C are illustrated at higher magnification in A'–C', respectively. Scale bar in A is 0.5 mm (B–C are at the same magnification).

more symmetrical callus formation observed in the absence of BMP2 beads (compare Figs. 5E, F with Figs. 1G, H).

Grafted blastema cells regenerated a critical-size defect

It is paradoxical that an entire radius can be regenerated if the arm is amputated; however, a critical-size defect was not regenerated in response to excision of the radial diaphysis. We hypothesized that the different responses to these two different injuries was a consequence of whether or not regeneration-competent blastema cells were induced. Blastema cells formed when a limb was amputated; whereas, callus formation in response to excision of the radius did not induce blastema cell formation, as reported above. To determine if blastema cells could regenerate an excised radius, we grafted mesenchymal cells from a medium bud blastema of a GFP donor animal whose arm had been amputated.

Grafted blastema cells survived and regenerated all or most of the excised radial cartilage in the host limbs (Figs. 6C, D; Table 1). We analyzed the regenerated limbs in whole-mount stained preparations (Fig. 6C), in which more than 60% of the defects were completely resolved (Table 1, $n = 7/11$), two were partially resolved, and two persisted. We sectioned limbs in which the defect was regenerated, and observed that the excised region was filled with differentiated chondrocytes that were contiguous with the cut ends of the radius (arrows in Fig. 6D). Although the gross morphology of the

regenerated cartilage was not the same as the host cartilage at this early time point (day 20 post-surgery), there was no evidence of scar tissue or defects between the graft and host cartilage. The regenerated cartilage was expanded laterally compared to the margins of the uninjured radius, and it is possible that with time, the regenerated segment of cartilage would be remodeled as occurs in mammalian bone repair (Schindeler et al., 2008). We did not section all of the limbs at the end of the experiment, and it is therefore possible that in those limbs with no regeneration, or incomplete regeneration, the number of grafted blastema cells that survived was reduced compared to the limbs in which the entire radial defect was repaired.

Most of the grafted blastema cells differentiated into chondrocytes and integrated into the cut ends of the host radius (Fig. 7A). Essentially all the GFP-positive donor cells were localized at the site of the excision and most expressed high levels of type-II collagen. The boundary between donor (regenerated) and host cells was sharp, and all cells at the interface expressed high levels of type-II collagen indicating that no fibrous tissues were formed between graft and host cells. Not all grafted cells differentiated as chondrocytes, and a few scattered GFP-positive cells were observed in the loose connective tissue surrounding the wound site. In histological sections, we observed some regions in which grafted cells associated with the regenerated radius did not express type-II collagen (Fig. 7A); however, in whole-mount preparations of these limbs, no gaps in the regenerated radius were observed (Fig. 6C). These grafted cells

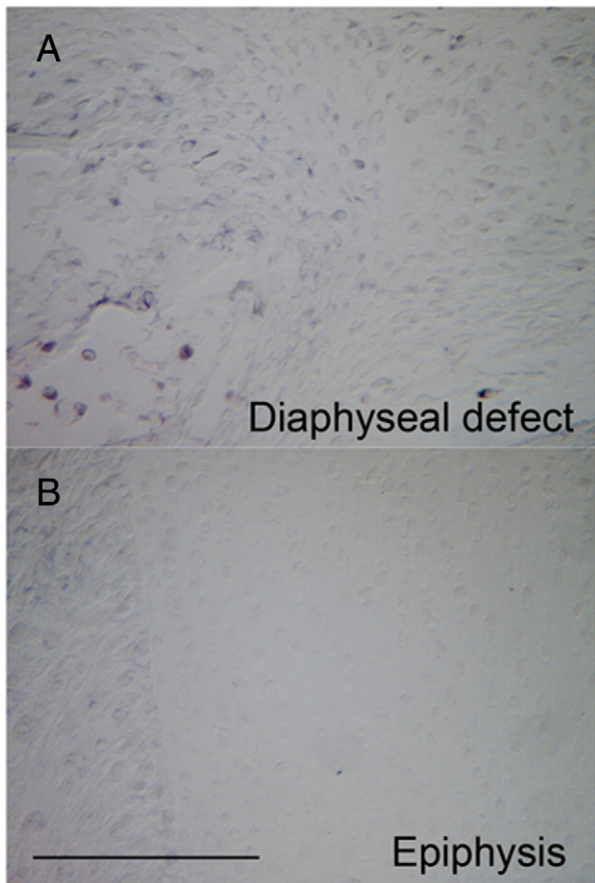


Fig. 4. BMP signaling in regenerating radial defects. Bmp-2 was expressed in cells associated with the callus at the cut edge of the radius (diaphyseal defect) at day 20 post-surgery (A), but not in cells associated with the ends of the radius (epiphysis) which was about 2 mm away from the injury site (B). Scale bars are 0.2 mm in B; A is at the same magnification.

presumably were still undifferentiated, or had formed connective tissues that were closely associated with the regenerated chondrocytes. As reported above, we detected high levels of expression of the blastema cell marker, *Prrx-1* in grafted blastema cells at this time point (Figs. 3C, C'), indicating that undifferentiated blastema cells were present in association with the regenerated defect.

In contrast to grafted blastema cells, differentiated chondrocytes and fibroblasts did not regenerate the excised radius. Chondrocytes were derived from dissociated articular cartilage, and fibroblasts were derived from the dermis of arm skin. Both types of cells survived in the host environment. Although dermal fibroblasts are the progenitors of blastema cells (Endo et al., 2004; Gardiner et al., 1986; Kragl et al., 2009; Muneoka et al., 1986), they did not regenerate the excised radius (Table 1; Figs. 6A, B). The excision wound was filled with fibrous tissue, and thus it appeared that the donor fibroblasts regenerated loose connective tissue but not chondrocytes (Fig. 6B). Similarly, grafted chondrocytes remained differentiated and formed cartilaginous nodules within the excision wound site (Figs. 6E, F). Although these nodules appeared to replace at least some of the excised radius (Table 1, $n=6/6$), they did not integrate into the ends of the host radius in contrast to grafted blastema cells (compare Figs. 6D and F). Unlike grafted blastema cells, grafted chondrocytes expressed only low levels of type-II collagen within the middle of the nodules, and the cells at the boundary between the graft and host did not appear to be chondrogenic (Fig. 7B). Thus neither fibroblasts nor chondrocytes were induced to dedifferentiate and become blastema cells in response to grafting into the excisional wound. Since blastema cells survived and regenerated the missing radial defect, the excisional wound

environment provided signals that were permissive for blastema cell maintenance, even though the signals to induce dedifferentiation and localized blastema cell formation were not present.

Dedifferentiated connective tissue fibroblasts regenerated a critical-size defect

It is known that non-skeletal cells in the stump have the ability to regenerate a missing skeletal element when the limb is amputated (Holder, 1989; Kragl et al., 2009; Lheureux, 1983; Muneoka et al., 1986), even if the skeleton is not present in the stump when the limb is amputated (Goss, 1969). Our results indicated that the signals to induce these cells to dedifferentiate and regenerate the skeleton were not present in the excisional wound environment. Given that signals from both the wound epithelium (WE) and the nerve are required to induce dedifferentiation and blastema formation in response to amputation (Satoh et al., 2008b), we tested whether these signals would also function in an excisional wound. Rather than closing the wound by reflecting the full-thickness skin flap, we removed the skin as well as the underlying muscle tissues to allow a WE to form over the excisional wound. We also surgically isolated the nerves of the lower arm and deviated them so as to lie in the middle of the wound where they came into contact with the wound epithelium after it formed. When a WE formed in the presence of a deviated nerve, the radial defect was completely regenerated in half the limbs by cells within the wound bed (Figs. 8C, D; Table 1, $n=4/9$).

Induced endogenous regeneration required signals from both the WE and a deviated nerve. When a deep wound was made to induce WE formation, but a nerve was not deviated, none of the limbs completely regenerated the radius (Table 1, $n=5$). Similarly, simple excisional wounds did not form a WE, and did not regenerate when a nerve was deviated (Figs. 8A, B; Table 1, $n=8$). Simple excisional wounds also did not regenerate when a nerve was not deviated (Fig. 1; Table 1, $n=14$). As expected, when the nerve supply to the entire limb was removed by denervation at the level of the shoulder simple excisional wounds did not regenerate the radial defect (Fig. 9; Table 1, $n=4$). Although deep wounds with a deviated nerve regenerated when the limb had an intact nerve supply, regeneration was inhibited when the limb was denervated proximally (Table 1, $n=3$). In simple excisional wounds in denervated limbs, callus formation appeared to be partially inhibited during the 20-day period post-surgery (compare Fig. 9 and Figs. 1G, H). Severed nerves regenerate and reinnervate a limb within 1 to 2 weeks, and thus callus formation may have been delayed until nerves regenerated back to the site of the wound. The relationship between nerve signaling and callus formation is being investigated further.

Excisional wounds with a WE and a deviated nerve formed blastemas that appeared to be equivalent to amputation-induced blastemas and to ectopic blastemas formed on the side of the arm (Satoh et al., 2007). We analyzed expression of blastema marker genes in excisional wounds induced to undergo endogenous regeneration 10 days post-surgery (Fig. 10). The excised epithelium had regenerated and was thickened in the region overlying the deep wound (Fig. 10A). The basal keratinocytes of the thickened epithelium expressed *Sp9*, a marker for the apical epithelial cap (AEC) of amputation-induced and ectopic blastemas (Satoh et al., 2008b). The epithelium formed over the deep wound thus appeared equivalent to the AEC of a regenerating limb blastema by both anatomical (locally thickened) and molecular (localized *Sp9* expression) criteria. Both blastema mesenchyme cell markers (*Prrx-1* and *Msx-2*) were expressed at high levels in cells within the defect (Figs. 10C, E) and in cells located between the radial defect and the overlying ectopic AEC (Figs. 10D, F). Although deep wounding combined with a deviated nerve appeared to induce formation of a blastema that regenerated the radial excision, none of the nine treated limbs formed an ectopic limb at the wound site.

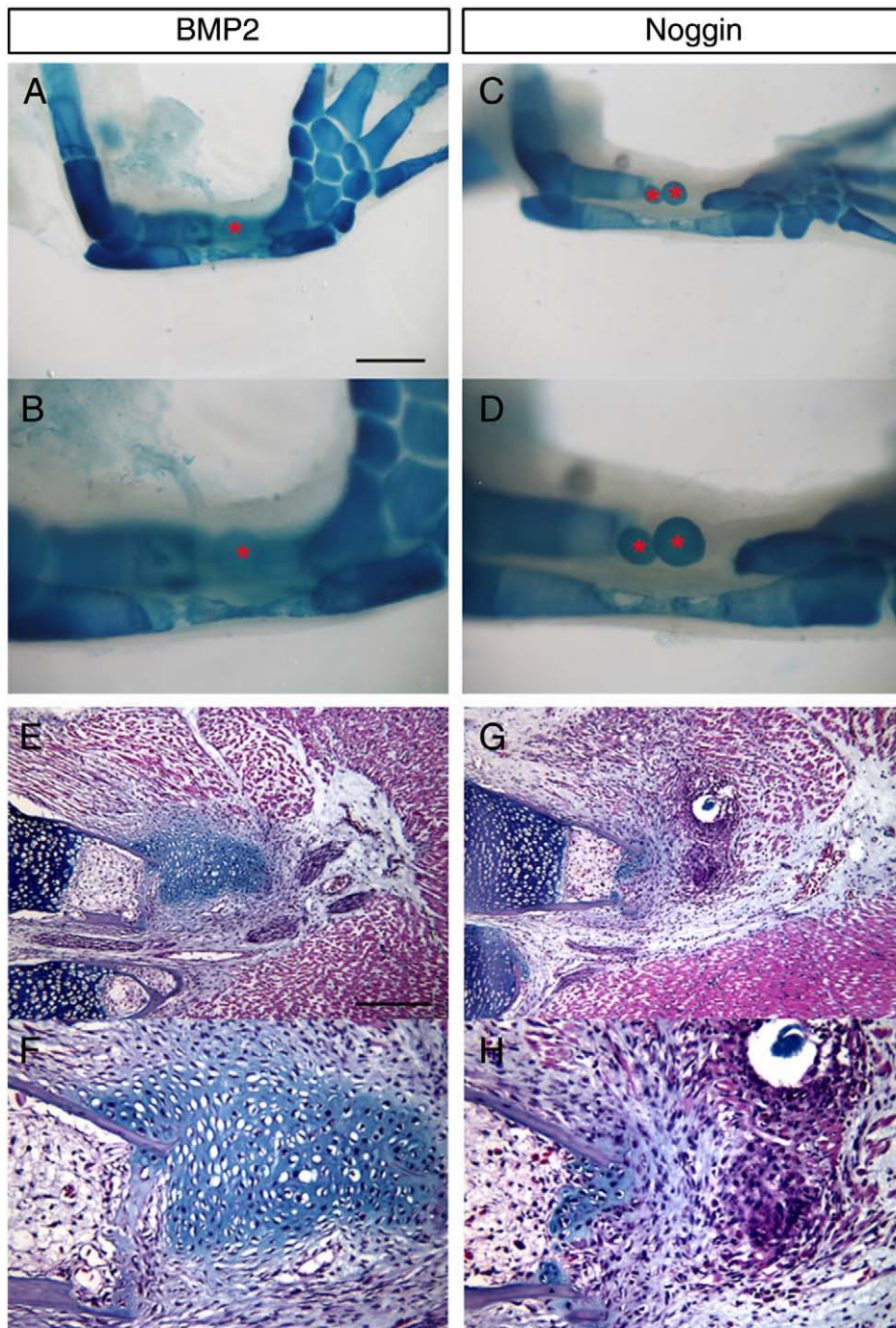


Fig. 5. Induced regeneration of a radial defect by BMP2. When beads soaked in BMP2 were implanted into the radial defect host site, chondrogenesis was stimulated and the defect was regenerated within 20 days post-surgery (A, B is at higher magnification). In histological sections, the regenerated cartilage was continuous with the cut end of the radius and was elongated along the proximal-distal axis of the limb (E, F is at higher magnification), in contrast to the cap-like callus that formed without BMP2 beads (Fig. 1). When beads soaked in Noggin were grafted into the defect (C, D is at higher magnification), callus formation was reduced at day 20 post-surgery (G, H is at higher magnification), as compared to untreated excisions (Fig. 1). The location of the grafted beads is indicated by red asterisks in A–D. Scale bars are 2 mm in A (C is at the same magnification) and 0.5 mm in E (G is at the same magnification).

To confirm that signals from the WE and deviated nerve induced connective tissue fibroblasts to dedifferentiate, we grafted dissociated dermal fibroblasts from a GFP transgenic donor into the radial defect of a deep wound with a deviated nerve. When fibroblasts were grafted into a simple excisional wound, they were localized within the connective tissue and did not contribute to the regenerated cartilage of the callus (Figs. 11E–G), and the defect persisted (Fig. 6A, B). In contrast, when grafted into a deep wound with a deviated nerve, the dermal fibroblasts contributed to the regenerated cartilage as well as the connective tissue (Figs. 11B–D), and

the defect was regenerated at a similar frequency as was observed when dermal fibroblasts were not grafted (Table 1). Grafted cells that remained in the connective tissue exhibited a fibroblastic morphology comparable to the adjacent host cells (Fig. 11D). In contrast, grafted cells that contributed to the regenerated cartilage exhibited the morphology of chondrocytes. These results are consistent with the conclusion that signaling from the WE and a deviated nerve induced fibroblasts in the wound environment to dedifferentiate to form blastema cells and contribute to the regeneration of the excised radius.

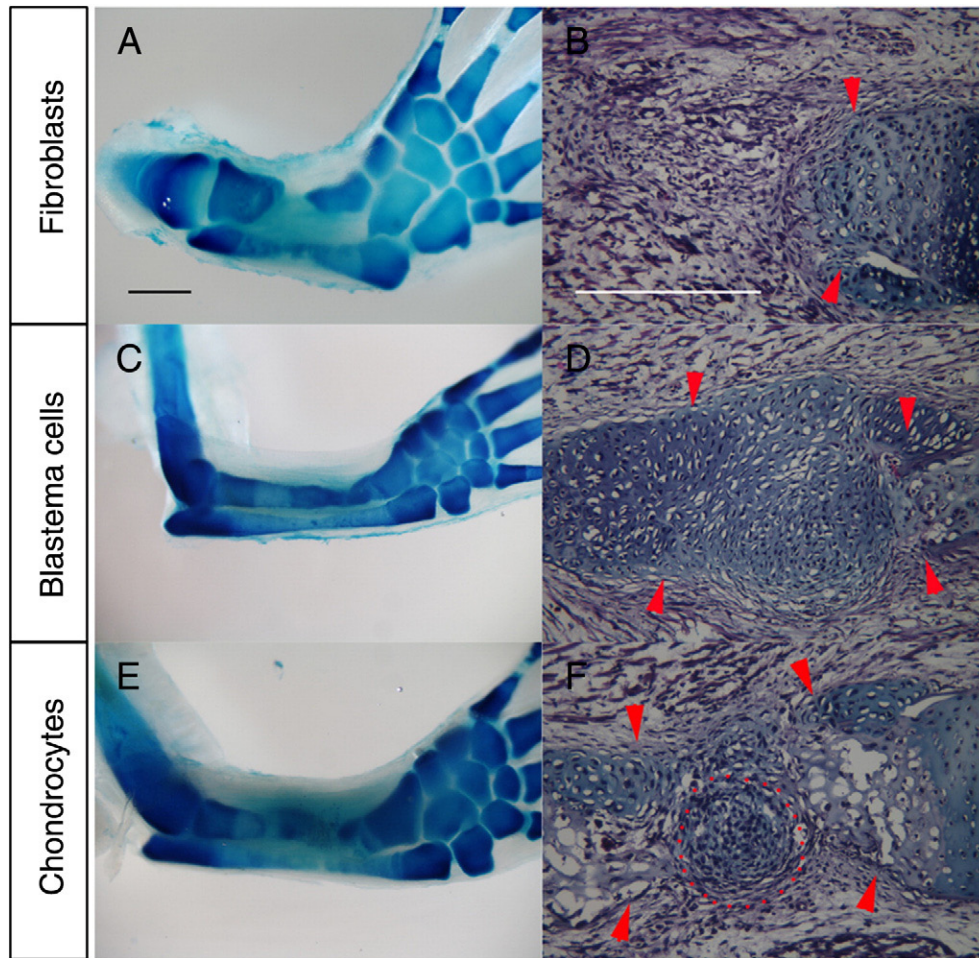


Fig. 6. Developmental fate of cells grafted into a radial defect. Dissociated dermal fibroblasts form loose connective tissue (B) but do not regenerate the missing cartilage in the radial defect (A). In contrast, grafted blastema cells differentiate as chondrocytes that integrate into the cut ends of the radius (D) and regenerate the radial defect (C). Chondrocytes derived from dissociated articular cartilage differentiate as chondrocytes within the excision, but form cartilaginous nodules (encircled by red dots in F) that do not integrate into the cut ends of the host radius (E, F). Red arrowheads indicate the cut ends of the host radius. Scale bars are 2 mm in A (C and E are at the same magnification) and 0.5 mm in B (D and F are at the same magnification).

Discussion

Salamanders and mammals have comparable regenerative responses to a skeletal defect

The loss of a length of bone above a threshold size, a “critical-size defect” (CSD), is not regenerated (Schmitz and Hollinger, 1986). This occurs in salamanders as it does in mammals and other vertebrates (Hutchison et al., 2007; this study), in spite of the ability of salamanders to regenerate an entire amputated limb. Unlike other vertebrates, a salamander does have the potential to regenerate the missing skeletal element, even if it typically does not. If the ulna is removed surgically and the limb is amputated, the ulna is regenerated distal to the amputation plane even though it was absent in the proximal stump at the time of amputation (Goss, 1969). Therefore, with the axolotl we know that regeneration can be induced if the appropriate signals are provided. In the absence of any additional signals, axolotl cells in the wound environment respond like mammalian cells and form a callus. In response to ectopic BMP treatment, this endogenous regenerative response is enhanced (Fig. 5). Finally, signals from the wound epithelium and nerve induce dedifferentiation of cells in the wound environment, leading to regeneration of the defect (Figs. 8, 10, 11). Our findings indicate that this induced regenerative response occurs by the same mechanisms that lead to blastema formation and limb regeneration in response to amputation.

The endogenous regenerative response to a skeletal excision in both axolotls and mammals is the formation of a cartilaginous callus that is mediated in part by BMP signaling (Schindeler et al., 2008; this study). When endogenous BMP signaling is enhanced by the delivery of exogenous BMP, the chondrogenic response is enhanced. This response to exogenous BMPs is not surprising since they are among the most important growth factors involved in the regulation of bone formation and growth (Reddi, 1998, 2005), and BMP2 and BMP7 are already approved for clinical therapies to induce bone formation. In the axolotl, treatment with BMP2 induces a directional outgrowth along the proximal-distal axis of the defect that regenerates the missing skeletal segment rather than just forming a larger callus. Therefore this response is not simply a non-specific induction of proliferation and chondrocyte differentiation, but a patterned response leading to restoration of the deleted anatomical structure. Amputations of neonatal mouse limbs also induce a hypertrophic cartilage response leading to callus formation (Masaki and Ide, 2007). Chondrogenesis is enhanced by exogenous BMP7 resulting in formation of structures appropriate to the proximal-distal level of the amputation, rather than a non-specific, enlarged callus (Masaki and Ide, 2007). Finally, mouse digit tips regenerate when amputated, and the regenerative response is dependent on BMP signaling (Han et al., 2003, 2008). Amputations at more proximal digit levels do not regenerate, but form a callus in neonatal mice.

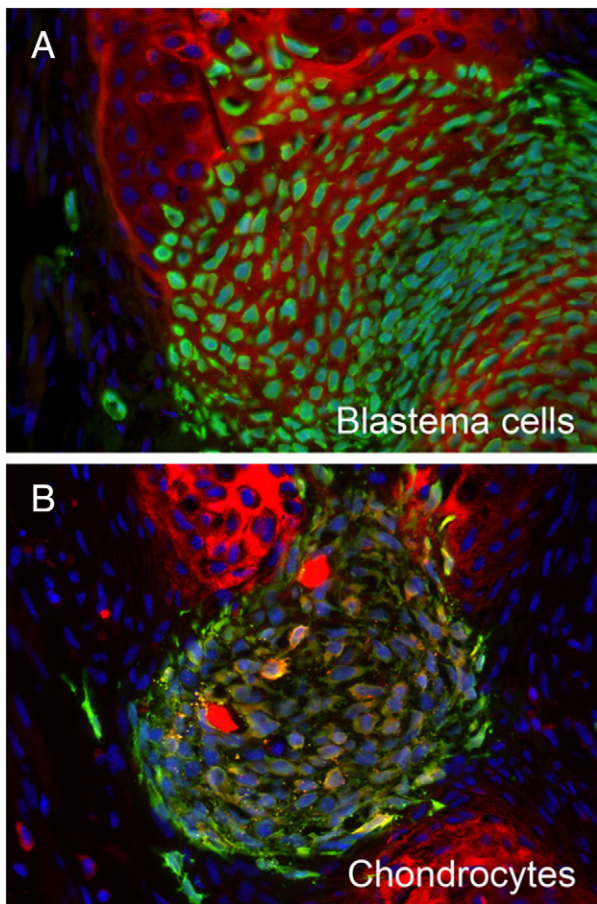


Fig. 7. Differentiation of blastema cells and chondrocytes grafted into a radial defect. Undifferentiated mesenchymal cells from a blastema induced by amputation of a limb of a GFP transgenic axolotl were dissociated and grafted into a radial defect (A). Twenty days post-surgery nearly all the grafted cells (green) had differentiated into chondrocytes as evidenced by the expression of type-II collagen (red). The distribution of both host and graft cells is visualized by DAPI staining of nuclei, and there is no discontinuity between the graft and host cells. In contrast, grafted chondrocytes derived from dissociated articular cartilage form nodules that express only low levels of type-II collagen (red) and do not integrate into the host cartilage (B).

Salamanders can regenerate a skeletal defect from non-chondrocyte progenitor cells

Although both axolotls and mice form a callus in response to an excisional wound, an axolotl can do more. When regeneration-competent cells are provided, the axolotl wound environment is permissive for regeneration of the excised skeletal element. The wound environment does not however induce cells to become regeneration-competent. Grafted chondrocytes form nodules that appear to differentiate as fibrocartilage rather than articular cartilage expressing high levels of type-II collagen (Fig. 7), a response that is comparable to what occurs when mammalian cartilage is injured. Most importantly, these chondrocytes do not integrate into the host skeletal elements. Similarly, grafted dermal fibroblasts form fibrous connective tissue (Fig. 6), even though these cells are the progenitor cells for blastema cells that could regenerate the limb cartilage when the limb is amputated. In contrast, when undifferentiated blastema cells are grafted into the wound, nearly all of them differentiate as chondrocytes, uniformly express high levels of type-II collagen, and integrate into the cut ends of the host skeleton (Figs. 6, 7). Since the majority of those cells were fated to give rise to connective tissues other than cartilage had they regenerated an amputated limb (Kragl et al., 2009; Muneoka et al., 1986; Tank and Holder, 1979), we conclude that signals in the wound environment altered their

developmental fate and directed them along the pathway leading to differentiated chondrocytes that regenerated the radial defect. Although signals to induce formation of regeneration-competent cells are not present, the axolotl wound environment is nevertheless permissive for regeneration, and is instructive for chondrocyte differentiation of multipotential, undifferentiated blastema cells.

The axolotl wound environment can be manipulated such that it also becomes inductive for blastema cell formation. In response to signals from the wound epithelium (WE) and a deviated nerve, the radial defect is regenerated by cells within the wound bed (Figs. 8, 10, 11). We presume that some portion of the regenerative response is a consequence of BMP-mediated proliferation and recruitment of chondrogenic cells as occurs in wounds without a WE and deviated nerve. However, that response is limited and localized to the cut ends of the radius, and does not involve blastema cell formation (Fig. 3). In contrast, the response to signals from the WE/nerve is widespread, and cells within the wound bed express the regeneration markers *Prrx-1* and *Msx-2* in the mesenchymal cells and *Sp9* in the basal keratinocytes of the WE (Fig. 10). Thus endogenous regeneration of the radial defect requires an early step involving the genesis of undifferentiated, multipotent cells from cells in the wound bed. Although we were not able to generate quantitative, cell contribution data from histological sections, at least some of the cells of the regenerated cartilage are derived by the induced dedifferentiation of connective tissue fibroblasts as evidenced by the contribution of grafted dermal fibroblasts to the regenerated cartilage in response to WE/nerve signals (Fig. 11).

Both ectopic limb blastemas and amputation-induced blastemas are derived in large part from progenitor cells within the loose connective tissues of the limb (Endo et al., 2004; Kragl et al., 2009; Muneoka et al., 1986). In both these types of limb regeneration, the early steps in the genesis of blastema cells are dependent on signals derived from the interaction of the WE and the nerve (Satoh et al., 2008b). Since endogenous regeneration of a radial defect also is dependent on these signals, we presume that the same mechanisms of regeneration in all three types of injuries (amputation, lateral skin wound and radial excision) function to replace the missing limb tissues and to restore the normal limb pattern. We also presume that these signals target the same population of blastema progenitor cells, loose connective tissue fibroblasts.

The function of connective tissue fibroblasts is to encode positional information within the limb (Bryant et al., 2002; Chang et al., 2002). In response to injury, they give rise to the early blastema cells that control growth and pattern formation, and ultimately regenerate the connective tissues of the limb. These functions are most dramatically and directly demonstrated by experiments in which a normally patterned limb (except that it lacks muscle) is regenerated from the dermis as the source of progenitor cells for the blastema (Holder, 1989; Lheureux, 1983). Similarly, cell contribution studies demonstrated that the early blastema is derived largely, if not entirely, from fibroblast progenitor cells (Bryant et al., 2002; Gardiner, 2005; Muneoka et al., 1986); a finding that has recently been confirmed (Kragl et al., 2009). Finally, of all the limb tissues, it is the connective tissue fibroblasts that regulate limb pattern formation. Grafts of tissues containing fibroblasts affect pattern formation, whereas tissues devoid of fibroblasts (e.g. cartilage) do not (Bryant et al., 2002; Gardiner, 2005; Muneoka et al., 1986). Of the fibroblast containing tissues, the dermis has a particularly dominant effect (see Bryant et al., 2002). The function of these cells in the control of pattern formation, has led to the hypothesis that during regeneration they form the blueprint that instructs the other cells of the limb as to where to migrate and differentiate in order to restore the integrated structure and function of the limb (see Gardiner, 2005). Thus during axolotl limb regeneration, fibroblasts have at least two functions; one is to provide positional clues within the regeneration-environment, and the other is to provide multipotential cells to regenerate the connective tissues.

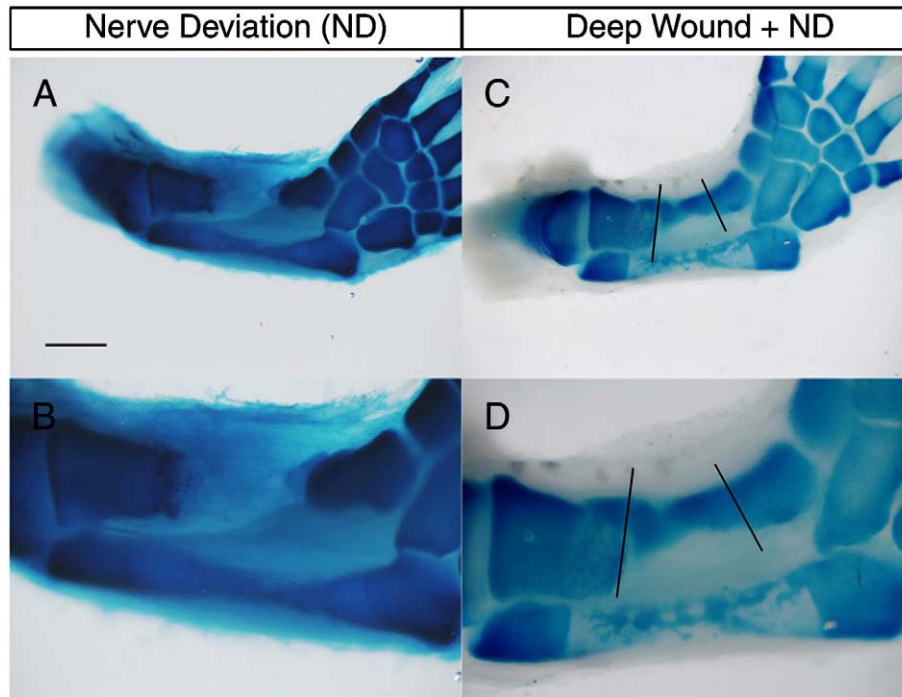


Fig. 8. Induced regeneration of a radial defect by signaling from a deviated nerve and a wound epithelium (WE). As observed with an untreated excision (Fig. 1), a critical-size radial defect persists when a nerve is deviated to the wound site (A, higher magnification in B). If a deep wound is created such that a WE forms over the radial excision and a nerve is deviated beneath the WE, the radial defect is regenerated (C, higher magnification in D). Lines indicate the position of the cut ends of the host radius (C, D). Scale bar = 2 mm (A, C is the same magnification).

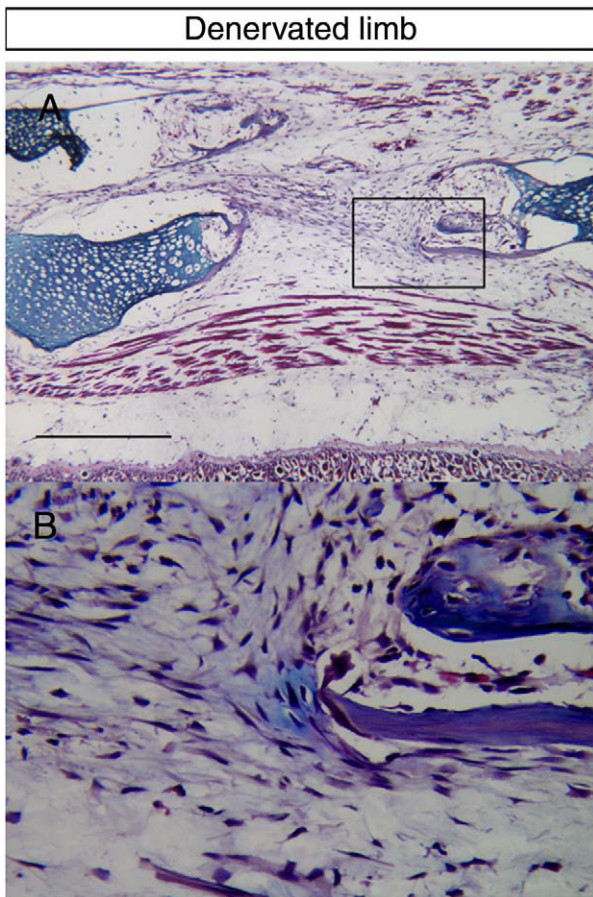


Fig. 9. Callus formation is nerve-dependent. Callus formation at day 20 post-surgery is inhibited when the limb is denervated by severing the brachial nerves where they enter the limb (A). The boxed area in A is illustrated at higher magnification in B. Scale bar = 1 mm.

WE/nerve signaling functions early in regeneration and is required for the dedifferentiation of cells and the activation of adult stem cells so as to reacquire the developmental potential of limb buds cells, as evidenced by the re-expression of embryonic genes (Han et al., 2005; Satoh et al., 2008a, 2008b). Once a blastema has formed, it appears by a variety of different criteria to be equivalent to the limb bud (see Bryant et al., 2002). From that point on, regeneration appears to be a recapitulation of development, leading to the later events associated with differentiation of limb cell types from a variety of progenitor cells. In some instances, the lineage relationship between the adult progenitor cells and redifferentiated cells at the end of regeneration is narrow and well defined. For example, regenerated muscle is derived from an adult stem cell population, the satellite cells (Cameron et al., 1986; Kragl et al., 2009; Morrison et al., 2006), and Schwann cells are derived from preexisting Schwann cells in the uninjured limb (Kragl et al., 2009). In the case of regeneration of the connective tissues, fibroblasts have the ability to reform a variety of tissues, including loose connective tissue, ligaments, tendons, muscle fascia, and cartilage (Holder, 1989; Kragl et al., 2009; Lheureux, 1983; this study). It therefore appears that WE/nerve signaling induces fibroblasts to revert to multipotential connective tissue progenitor cells characteristic of developing limb buds, and that these cells then regenerate the various connective tissues, including cartilage. This dynamic, developmental plasticity of fibroblasts is an essential feature of regeneration.

Regeneration is a multistep process

Our findings emphasize that regeneration is not simply about inducing differentiation of a missing cell type, e.g. chondrocytes to regenerate a radial defect. Successful regeneration requires both the availability of regeneration-competent cells and the creation of an environment that is both permissive and instructive for those cells to restore the missing tissues. Although chondrogenic cells can remake the missing cartilage, the information specifying where they are to do that appears to be encoded in the surrounding environment. In the

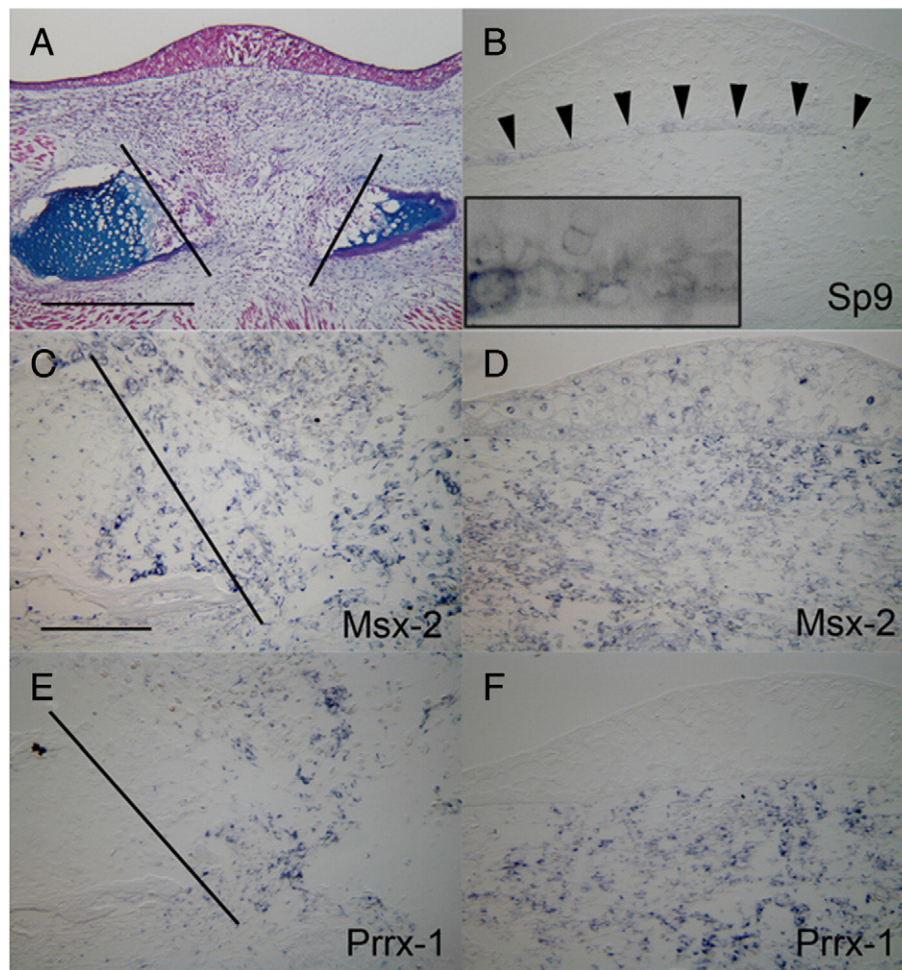


Fig. 10. Induction of blastema cells in a radial excision wound. A deep wound was created to allow a wound epithelium to form, and a nerve was surgically deviated to the radial defect. At day 10 post-surgery, a thickened epithelium had formed in the region of the radial deletion (A) (H&E staining). The basal keratinocytes of the thickened epithelium expressed *Sp9* (B, insert is a higher magnification view), which is a marker for the apical epithelial cap formed at the distal tip of amputation-induced and ectopic blastemas. The cells beneath the thickened epithelium expressed the blastema marker genes *Msx-2* and *Prrx-1* at high levels (D, F). Cells within the radial deletion also expressed *Msx-2* and *Prrx-1* at high levels (C, E). Lines (A, C and E) indicate the position of the cut ends of the host radius. Scale bar is 1 mm in A and 0.3 mm in C, (B–F are at the same magnification).

axolotl radial defect model, BMP signaling targets cells with chondrogenic potential, but does not induce cells such as fibroblasts to acquire that potential. The ability of fibroblasts to revert to a multipotential, more embryonic state requires an earlier set of signals from the WE/nerve to reprogram these cells such that they can respond to appropriate morphogenetic signals (e.g. BMP) in order to remake the missing tissues (e.g. the cartilage of the radial defect). It thus appears that two different signaling pathways (BMP and WE/nerve) target different populations of cells at different steps in regeneration. At a later step, BMP signaling targets cells (perichondrocytes or multipotent blastema cells) to induce chondrogenesis, whereas at an earlier step, WE/nerve signaling targets connective fibroblasts to dedifferentiate and become responsive to BMP signaling.

Although the complete regenerative response in the axolotl involves both the genesis of blastema cells and the induction of the regeneration niche, it appears that in the absence of the former, the formation of the permissive and instructive regeneration environment still occurs. When blastema cells induced by limb amputation are grafted into the excision, they survive and are induced to regenerate the missing radial cartilage that integrates into the host cartilage. We cannot rule out the possibility that the grafted blastema cells actually induce their own regeneration niche. However, the patterned cartilage induced by ectopic BMP is consistent with the hypothesis that the regeneration niche is induced by injury even if fibroblast dedifferentiation is not. This

view is also consistent with the classic observation that an amputated limb without an ulna regenerates an ulna distally, but not proximally (Goss, 1969). The information for the pattern of the skeleton is encoded in the stump tissues rather than the skeleton itself, and when combined with blastema cells the ulna is regenerated distally; whereas, the lack of blastema cells proximally results in the persistent ulna defect (Goss, 1969).

Although the axolotl has the ability to carry out all the steps required for a complete regenerative response, it is possible experimentally to study the steps in isolation from one another. Previous studies of ectopic limb formation in the axolotl (the Accessory Limb Model) have demonstrated that there are multiple steps involved in formation a limb *de novo*, and that failure to progress from one step to the next step results in a failure to regenerate an entire limb (Endo et al., 2004; Satoh et al., 2007). In the present study, we have demonstrated that an excisional wound also exhibits a range of regenerative responses, depending on the signaling pathways that are activated. By studying individual steps in isolation from the whole, one might get the impression that there are different types of regeneration (e.g. callus formation versus radius regeneration). An alternative interpretation is that when only part of the regeneration pathway is activated, the regenerative response is incomplete. By this view, discussions about whether a regenerative response is “tissue regeneration” or “epimorphic regeneration” (Carlson, 1978; Goss and Holt, 1992; Korneluk and Liversage, 1984) are a distinction without a

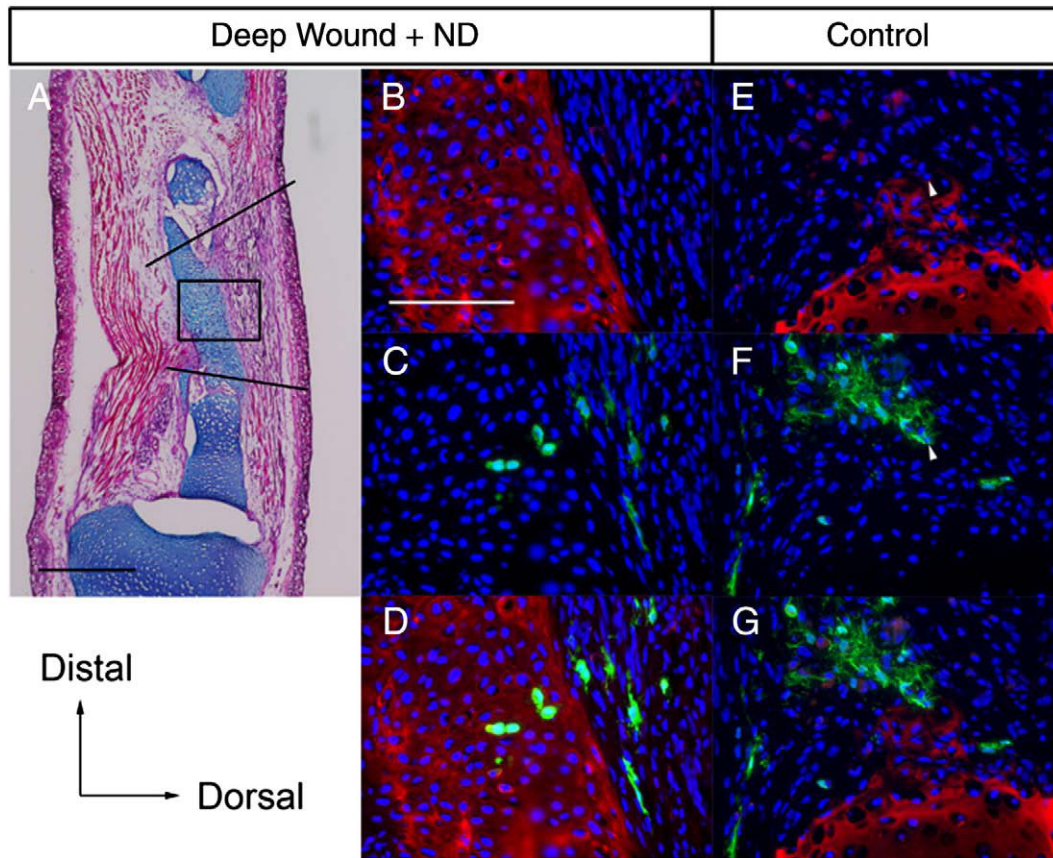


Fig. 11. Reprogramming of dermal fibroblasts to contribute to cartilage regeneration. Dissociated dermal fibroblasts were isolated from a GFP transgenic donor animal and grafted into an excisional wound that had been induced to regenerate (Deep Wound + ND) or a control excision (Control) that formed a callus as in Fig. 1. In control wounds (E–G), the grafted cells (green) contributed to the loose connective tissue, but not to the regenerated cartilage of the callus (red, type-II collagen immunohistochemistry). In contrast, signals from the wound epithelium of the deep wound and the deviated nerve induced dedifferentiation of cells within the wound bed, including the grafted dermal fibroblasts. When induced to dedifferentiate, the grafted cells (green) contribute to both the regenerated connective tissues and to the regenerated cartilage of the radius (A–D). The regenerated radius is illustrated in A (H&E staining), in which the lines indicate the position of the cut ends of the host radius, and the box indicates the region illustrated at higher magnification in B–D. The limb is oriented with distal towards the top of the image and dorsal to the right. Type-II collagen expression is illustrated in B and E, GFP positive cells are illustrated in C and F, and the two images are merged in D and G. DAPI-stained nuclei are illustrated in all the images. Scale bar is 1 mm in A and 0.3 mm in B, (C–G are at the same magnification).

difference. In contrast, we view regeneration as a genetically conserved, multistep process that can be manipulated to yield a complete and perfect regenerative response as evidenced in the axolotl.

Finally, the interactions between undifferentiated, multipotential blastema cells and “blueprint” cells within the regeneration niche indicate the feasibility of cell-based therapies for tissue repair and regeneration. In the case of the axolotl, the regeneration-competent cells can be generated intrinsically by the reprogramming of connective tissue fibroblasts by WE/nerve signaling. In mammals, it is now evident that multipotent, regeneration-competent cells can be generated from embryonic stem cells or induced pluripotent stem cells. The key to utilizing these cells lies in the ability to direct their developmental fate within the wound environment. The challenge is to understand how to manipulate this host environment so as to create the information blueprint for regeneration of the missing structures. The Excisional Regeneration Model in the axolotl provides a tool to identify and manipulate the critical signals that regulate the behavior of the connective tissue fibroblasts leading to formation of the regeneration niche.

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