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Nerve-induced ectopic limb blastemas in the axolotl are equivalent to amputation-induced blastemas

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Abstract

Adult urodeles (salamanders) are unique in their ability to regenerate complex organs perfectly. The recently developed Accessory Limb Model (ALM) in the axolotl provides an opportunity to identify and characterize the essential signaling events that control the early steps in limb regeneration. The ALM demonstrates that limb regeneration progresses in a stepwise fashion that is dependent on signals from the wound epidermis, nerves and dermal fibroblasts from opposite sides of the limb. When all the signals are present, a limb is formed *de novo*. The ALM thus provides an opportunity to identify and characterize the signaling pathways that control blastema morphogenesis and limb regeneration. Our previous study provided data on cell contribution, cell migration and nerve dependency indicating that an ectopic blastema is equivalent to an amputation-induced blastema. In the present study, we have determined that formation of both ectopic blastemas and amputation-induced blastemas is regulated by the same molecular mechanisms, and that both types of blastema cells exhibit the same functions in controlling growth and pattern formation. We have identified and validated five marker genes for the early stages of wound healing, dedifferentiation and blastema formation, and have discovered that the expression of each of these markers is the same for both ectopic and amputation-induced blastemas. In addition, ectopic blastema cells interact coordinately with amputation-induced blastema cells to form a regenerated limb. Therefore, the ALM is appropriate for identifying the signaling pathways regulating the early events of tetrapod limb regeneration.

Keywords: Axolotl; Limb; Regeneration; Blastema; Fibroblast; Dedifferentiation; Hoxa-13; Msx-2; Prx-1; Tbx-5; Mmp-9

Introduction

Adult urodeles (salamanders) are unique in their ability to regenerate complex organs perfectly. Given the conservation of genetic mechanisms among vertebrates, it is likely that the cellular and molecular processes that regulate urodele limb regeneration are shared among all vertebrates. Since other vertebrates do not exhibit the same extent of organ regeneration, presumably these basic regenerative mechanisms are not activated in response to injury. Therefore, an understanding of how urodele regenerative mechanisms are regulated will be directly applicable to understanding how to induce an enhanced regenerative response in other vertebrates, including humans. For this reason, salamanders are an important model organism for studies of tissue and organ regeneration.

Urodele limb regeneration is the most extensively studied and best understood model of tetrapod regeneration. In response to amputation, cells from limb tissues around the amputation surface dedifferentiate and give rise to a mass of undifferentiated and proliferating cells, the blastema cells, which eventually reform the missing limb structures. Although the events that are triggered by amputation and that lead to blastema formation have been extensively studied and described (Bryant et al., 2002; Gardiner et al., 2002), it has proved challenging to identify the specific molecular events that are necessary and sufficient for limb regeneration. Because amputation does extensive damage to all the limb tissues, until recently it has not been possible to distinguish regulatory events essential for regeneration from extraneous processes associated with trauma, inflammation, and necrosis.

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The recently developed Accessory Limb Model (ALM) provides an opportunity to identify and characterize the essential signaling events that control the early steps in limb regeneration (Endo et al., 2004). The phenomenon of accessory (ectopic) limb formation was discovered and studied repeatedly in the mid to late 20th Century (Bodemer, 1958, 1959; Egar, 1988; Lheureux, 1977; Maden and Holder, 1984; Reynolds et al., 1983), but only recently has its utility as an experimental model been realized (Endo et al., 2004). Briefly, the ALM has identified three major steps in limb regeneration. During the first step, the skin wound heals, and in the absence of additional signals the skin regenerates without scar formation. However, if the nerve supply to the wound is augmented by deviating a nerve to the wound site, dermal fibroblasts are induced to dedifferentiate and form an ectopic blastema. In the absence of additional signals, the ectopic blastema eventually stops growing and fails to form an ectopic limb. However, if dermal cells from the opposite side of the limb are grafted to the wound site in conjunction with deviation of a nerve, the induced ectopic blastema progresses to form a limb *de novo* in response to positional signaling between the progeny of the grafted dermal cells.

The ALM demonstrates that an excisional skin wound, a skin graft and a deviated nerve are sufficient for activating the limb formation pathway that is shared by all tetrapods. Since there is no damage to the underlying soft tissues, the processes of initiation, progression, and completion of *de novo* limb formation can be studied in the absence of the multitude of extraneous events associated with the trauma of amputation. Thus, during the early stages of regeneration, it is possible to isolate dermal cells as the target cell population, and the nerve and wound epidermis as the source of the signals for dedifferentiation and blastema formation. At the same time, the ALM demonstrates that a nerve and wound epidermis are not sufficient for making an entire new limb. It is during the steps following morphogenesis of the blastema that signals from the progeny of the dermal cells regulate the cell–cell interactions leading to pattern formation and sustained growth of the accessory limb. Most importantly, the ALM provides a positive assay to identify the steps leading from wound healing to blastema formation, and eventually to limb regeneration.

The original report on the ALM provided data on cell contribution, cell migration, and nerve dependency indicating that an ectopic blastema is equivalent to a blastema that is formed on an amputated limb (Endo et al., 2004). It therefore is likely that the same molecular mechanisms control dedifferentiation, blastema formation, and limb formation in both models for limb regeneration. In this study, we have extended the comparison of ectopic limb formation and limb regeneration to include an analysis of the underlying molecular mechanisms. To this end, we have identified five markers for the early stages of wound healing, dedifferentiation and blastema formation. Expression of each of these markers is the same for the induction of both ectopic and amputation-induced blastemas. In addition, we report that ectopic blastema cells interact coordinately with amputation-induced blastema cells to form a regenerated limb. Therefore, the ALM is appropriate for identifying the signaling pathways regulating the early events of tetrapod limb regeneration.

Methods and materials

Animals

Experiments were performed on albino or white axolotls (*Ambystoma mexicanum*) spawned at either the University of Kentucky Ambystoma Genetic Stock Center or at the University of California Irvine. Larvae (10–15 cm, snout to tail tip) were maintained at 21–23 °C in 20% Holtfreter's solution. Animals were anesthetized in 0.1% MS222 for surgical procedures.

Surgical procedures

Surgical procedures for the creation of lateral wounds, ectopic blastemas, and accessory limbs are described in detail in Endo et al. (2004). Briefly, wounds were created by removing a square of skin (1.0–1.5 mm on a side) from the anterior or posterior of the mid-upper arm, making sure that the underlying muscle is not damaged. An ectopic blastema was induced by surgically deviating the brachial nerve beneath the skin to bring the cut end of the nerve to the center of the skin wound. To induce an ectopic (accessory) limb, we additionally grafted a piece of skin removed from the contralateral side of the limb on the same animal to the wound site on the opposite side of the host limb. We typically made the host wounds on the anterior side of the limb and grafted a piece of posterior skin; however, the reciprocal grafting experiment yielded the same results (Endo et al., 2004). Samples of early stage ectopic blastemas were collected 5 days after the surgery, and late stage samples were collected 15 days after the surgery.

The ability of ectopic blastema cells, amputation-induced blastema cells and head skin cells to participate in regeneration was tested by grafting these cells into a host site at the base of a medium bud blastema. To do this, donor tissues (10 day ectopic blastemas, epidermis-free medium bud blastema, or head skin) were dissociated using 0.25% trypsin/PBS. The suspension of dissociated cells was neutralized with 10% FBS-containing culture medium and then washed with serum free DMEM. To label the cells prior to grafting, we used the fluorescent dye, PKH26 (Sigma) according to the manufacturer's instructions. Serum containing medium was added to stop the labeling reaction and the cells were washed with PBS. Cells were aggregated into a fibrin-clot composed of 25 mg/ml of fibrinogen (Sigma) and 200 U/ml of Thrombin (Sigma) (Groell et al., 1993; H. Ide, personal communication). The graft of the cells within the fibrin clot was placed beneath the epidermis in the central region of a medium bud blastema, and the fate of the cells was imaged every other day until the host limb had completed regeneration.

For experiments to determine if ectopic blastema cells had positional information, ectopic blastemas were induced to form on either the anterior or posterior side of the upper arm and were collected at 10 days after the operation. The hindlimb of each donor animal was also amputated to form a medium bud blastema into which the ectopic blastema cells could be grafted without being rejected by the host immune system. Ectopic blastema cells (either anterior or posterior) were grafted as above, except that they were grafted beneath the epidermis on the anterior side of the host blastema.

Samples for analysis of skeletal patterns were fixed in 10% neutral-buffered formalin overnight, rinsed in PBS, stained with 0.1% Alcian blue in 70% ethanol with 0.1 N HCl at 37 °C overnight, and then treated overnight in 4% KOH. Finally, the tissues were cleared in 50% glycerin/2% KOH for several days and stored in 100% glycerin.

RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy Kit (Qiagen) according to the manufacture's protocol. We used (dT)12–16 as a primer to reverse transcribe

cDNA from total RNA (100 mg) with Superscript III (Invitrogen) according to the manufacture's protocol. The reaction mixtures were diluted with TE and used as template for PCR. Primers for RT-PCR were designed as follows:

Msx2 forward: CACCCCTTTCACCACCTCCC
Msx2 reverse: AAGGGTAAACTGAAGCCAGG
Hoxa-13 forward: TCGGATGCGAACTCGTACAG
Hoxa-13 reverse: CATTAGCTGGTGGTCTTGAG
AmTbx-5 forward: ACGATTATACGTCCATCCGG
AmTbx-5 reverse: AAGCAGTCTCCGAGAACACG
AmPrx-1 forward: GGCGAAAGTTTGCTCTTCGG
AmPrx-1 reverse: CGGCGAAACAGGACCACCTT
Ef-1α forward: AACATCGTGGTCATCGGCCAT
Ef-1α reverse: GGAGGTGCCAGTGATCATGTT

PCR reactions were carried out using rTaq (Takara) for 32 cycles with the following conditions: 96 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s. *Ef-1α* cDNA was amplified for 26 cycles with the same cycling program. PCR products were loaded and resolved in 2% agarose gels.

Cloning of AmTbx-5 and AmPrx-1

Partial clones of axolotl *AmTbx-5* and *AmPrx-1* were isolated by RT-PCR using the following degenerate primers:

AmTbx-5 forward, 5′-CAGGGMATGGARGGRATYAA-3′
AmTbx-5 reverse, 5′-TACATGCARGCYTGICKCTG-3′
AmPrx-1 forward 5′-CAGMTSAAYKCWGARSAGAAG-3′
AmPrx-1 reverse, 5′-CCCCAGGASAGRTATCRKTGG-3′.

First-strand cDNA was synthesized from total RNA from forelimb blastemas and limb buds as detailed above. The PCR reactions were performed using rTaq (Takara) with the following conditions; 96 °C for 20 s, temperatures ranging from 42 °C to 60 °C for 20 s, 72 °C for 1 min. The PCR reaction products were purified by gel electrophoresis, cloned into pCRII-TOPO cloning vector (Invitrogen) using the manufacture's protocol, and sequenced.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described previously (Gardiner et al., 1995) with the following modifications. Samples were treated with proteinase K (10 μg/ml) at room temperature for 25 min, and riboprobes were hybridized at 60 °C. Following hybridization, tissues were washed electrically (Endo et al., 1997). Staining of all specimens was done with BCIP and NBT in alkaline phosphatase buffer as in the original protocol. In situ hybridization to tissue sections was performed as described previously (Satoh et al., 2006).

Results

Isolation of axolotl Prx-1 (AmPrx-1) and Tbx-5 (AmTbx-5)

Previous studies have implicated *Msx2, Matrix metalloproteinase-9* (*Mmp-9*) and *Hoxa-13* as being important regulators of axolotl limb regeneration (Carlson et al., 1998; Gardiner et al., 1995; Kato et al., 2003; Koshiba et al., 1998; Park and Kim, 1999; Yang et al., 1999). To identify and validate additional axolotl blastema marker genes, we used degenerate RT-PCR to clone the axolotl homologs of *Prx-1* and *Tbx-5*. We designed primers based on regions of identified conservation among multiple species, and we were able to clone the axolotl homologs of both these genes (Fig. 1). *A. mexicanum Prx-1* (*AmPrx-1*) is a partial clone of 345 nucleotides that includes the homeodomain (indicated in red font in Fig. 1B). When compared to sequence data for *Prx-1* for other species, *AmPrx-1* has the highest degree of identity to *Xenopus Prx-1* at the conceptual amino acid level for the entire clone (97%); and to zebrafish, chick, and *Xenopus* within the homeodomain (100%).

We also isolated a 1000 nucleotide partial clone of the *A. mexicanum* homolog of *Tbx-5* (*AmTbx-5*, Figs. 1C, D) that contains the T-box domain (indicated in red font in Fig. 1D). The sequence of *AmTbx-5* has a high degree of identity with the newt (*Notophthalmus viridescens*) homolog (87% at the nucleotide level and 93% at the amino acid level for the entire clone, and 98% amino acid identity within the T-box).

Expression of AmPrx-1 and AmTbx-5 in limb development and limb regeneration

To investigate the expression of these newly cloned axolotl genes during limb development, we performed whole-mount *in situ* hybridization for multiple stages of developing forelimb buds (Fig. 2). Expression of both *AmPrx-1* and *AmTbx-5* was first detected in the flank region prior to the earliest evidence of an outgrowth of the limb bud (Figs. 2A, E). This expression was maintained as the limb developed, and was restricted to the limb bud (Figs. 2B–D and F–H). Both genes were expressed throughout the early limb bud (Figs. 2B and F), and at later stages they were expressed at higher levels in proximal regions (Figs. 2C, D and G, H). Neither gene appeared to be expressed in the epidermis (most evident in Figs. 2C, D and F–H). Thus, *AmPrx-1* and *AmTbx-5* are markers for mesenchymal cells of the developing limb, as has been demonstrated for a number of other species (Isaac et al., 1998; King et al., 2006; Logan et al., 2002; Ng et al., 2002; Nohno et al., 1993; Suzuki et al., 2005).

The expression patterns of *AmPrx-1* and *AmTbx-5* were also similar to each other during limb regeneration induced by amputation (Figs. 3 and 4). Both genes were expressed uniformly in mesenchymal cells of the early blastema (Figs. 3J, K) and continued to be expressed uniformly at later stages of regeneration (Figs. 4J, K). There was not an observable difference in the level of expression along the proximal–distal axis that was comparable to what was observed in whole-mount preparations of late stage developing limb buds. We note that expression of both of these genes, as well as *Msx-2*, could be detected by RT-PCR (see below) in mature skin prior to amputation. Therefore, expression of *AmPrx-1* and *AmTbx-5* (as well as *Msx-2*) was not induced *de novo*, but rather was rapidly upregulated in response to amputation. Upregulated expression of *AmPrx-1* and *AmTbx-5* was restricted to the blastema mesenchyme; these genes were not expressed in the epidermis of the blastema (Figs. 3J, K and Figs. 4J, K).

The three other regeneration marker genes that we analyzed in the ALM, *Mmp-9*, *Hoxa-13*, and *Msx-2*, have been characterized in previous studies. Expression of these genes was originally analyzed by whole-mount *in situ* hybridization, and it was demonstrated that all three are expressed during the early stages of limb regeneration. In order to allow for an

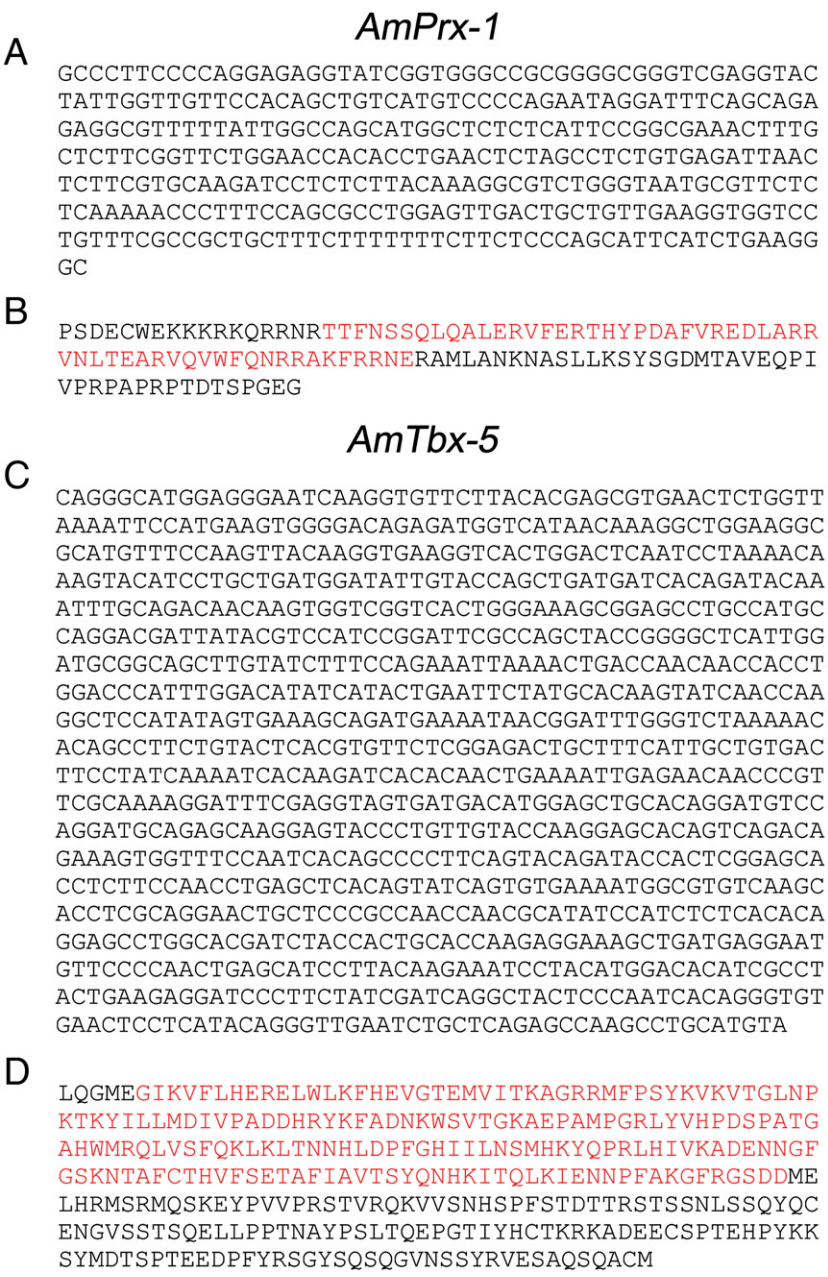

Fig. 1. Axolotl (*Ambystoma mexicanum*) *Prx-1* and *Tbx-5* DNA nucleotide sequences and deduced amino acid sequences. *AmPrx-1* DNA sequence (A) and deduced amino acid sequence (B). Red highlighted amino acid sequence in panel B indicates the DNA-binding domain (homeodomain). *AmTbx-5* DNA sequence (C) and deduced amino acid sequence (D). Red highlighted amino acid sequence in panel D indicates the T-box domain.

analysis of the coordinated expression of these genes for a single tissue sample, along with *AmPrx-1* and *AmTbx-1*, we conducted an *in situ* hybridization analysis of histological sections of wounds and amputation-induced blastemas.

Mmp-9 was one of the earliest expressed genes in response to amputation. It was initially expressed in the migrating wound epidermis within 2 h of wounding, which is prior to reepithelialization which takes about 4 h (Yang et al., 1999). Its expression persisted in the wound epidermis for the first two to 3 days of regeneration in the same pattern as observed at 24 h (Fig. 5H), after which it was no longer expressed in the epidermis (Yang et al., 1999). *Mmp-9* expression was also induced in skin wounds without an amputation; however, it was more rapidly downregulated and was not detected at 24 h post-wounding (Fig. 5E). Beginning at 24 h, *Mmp-9* was also expressed in association with the distal tips of the amputated skeletal elements (Fig. 5H). Expression in this region increased over the next couple of days and persisted throughout the period of blastema formation (Yang et al., 1999).

Msx-2 expression was also upregulated in the wound epidermis prior to the completion of reepithelialization (Carlson et al., 1998). *Msx-2* was detected weakly in epidermal cells of intact limbs (data not shown); a finding that was confirmed by RT-PCR (see below). In contrast to *Mmp-9*, *Msx-2* was expressed in both the wound epidermis and the underlying mesenchymal cells of simple skin wounds

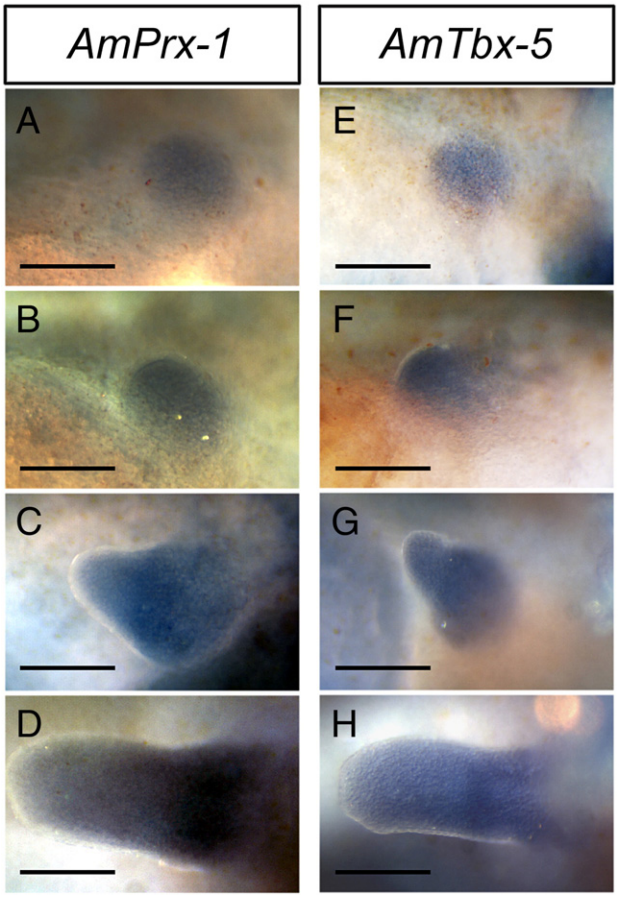

Fig. 2. Expression of *AmPrx-1* and *AmTbx-5* in developing axolotl forelimb buds. *AmPrx-1* and *AmTbx-5* were expressed in the flank region prior to the onset of limb bud outgrowth (A and E). Both genes were expressed throughout the early limb bud (B and F) but were expressed at higher levels proximally at later stages (C, D and G, H). Neither gene was expressed in the epidermis, as is particularly evident in panels C, D, F–H. Limb buds are oriented such that anterior is down and distal is toward the left. Scale bars = 200 μm.

(Fig. 5A, insert). Although expression of *Msx-2* did not persist in simple skin wounds, an elevated level of expression was still observed at 24 h after wounding (Fig. 5A). Expression of *Msx-2* persisted throughout all the stages of regeneration of an amputated limb (Figs. 3L, 4L, and 5D). Although expression was higher in the epidermis relative to the mesenchyme during wound healing and early stages of dedifferentiation, epidermal expression decreased and mesenchymal expression increased during blastema morphogenesis and growth. Consequently, by early-medium bud (Fig. 3L), and more so by late bud (Fig. 4L) blastema stages, *Msx-2* was expressed at high levels in the mesenchymal cells of the blastema, but was only weakly detected in the epidermis. It was reported previously, based on whole-mount *in situ* hybridization data (Carlson et al., 1998), that expression was stronger in the epidermis and weaker in the blastema mesenchyme. We consider it likely that the previous findings reflected tissue preparation and hybridization procedures that optimized detection of epidermal expression at the expense of the detection of mesenchymal expression. For that reason, the current data from hybridization to tissue sections are a more accurate demonstration of the spatial and temporal expression of *Msx-2* during limb regeneration.

Hoxa-13 was not expressed in mature skin, and its expression was induced in stump cells near the amputation plane within 24 to 48 h after amputation (Gardiner et al., 1995). As reported based on data from whole mount *in situ* hybridization (Gardiner et al., 1995), *Hoxa-13* was expressed uniformly during early stages of regeneration, up to and including the early bud blastema (Fig. 3I), but as the blastema grew, *Hoxa-13* expression became more restricted to the distal region that eventually regenerated the autopod (Fig. 4I).

Ectopic blastemas and accessory limbs express the same marker genes for dedifferentiation and regeneration as do amputation-induced blastemas

Since ectopic blastemas can be induced to form a limb *de novo*, they appear to be equivalent to the blastema formed on an amputated limb stump. To determine if the same genes are expressed during ectopic blastema formation and ectopic limb formation as are expressed during regeneration of an amputated limb, we used *in situ* hybridization to compare the expression of several marker genes in a simple skin wound, an ectopic blastema (wound plus deviated nerve), an ectopic limb (wound plus deviated nerve plus contralateral skin graft), and an amputation-induced limb blastema. For each gene analyzed (*AmPrx-1*, *AmTbx-5*, *Mmp-9*, *Msx-2*, and *Hoxa-13*), the pattern of expression in ectopic blastemas/limbs and amputation-induced regenerates was the same. In addition, the pattern of expression for each was the same for ectopic blastemas either with or without a skin graft. Thus, the expression of each of the dedifferentiation/blastema marker genes was induced in response to factors provided by the wound epidermis and the grafted nerve and was not dependent on interactions between anterior and posterior skin derived cells.

AmPrx-1 and *AmTbx-5* were expressed in all the mesenchymal cells throughout both ectopic blastemas (Figs. 3B, C; Figs. 4B, C) and ectopic limbs (Figs. 3F, G; Figs. 4F, G). This uniform expression pattern was comparable to what was observed in amputation-induced blastemas (Figs. 3J, K; Figs. 4J, K). Although an ectopically induced limb eventually formed a remarkably complete and normal limb pattern (Endo et al., 2004), at the early stages of development illustrated in Fig. 4, the ectopic blastema cells were more localized in the center of the blastema as compared to the more uniform distribution of cells in an amputation-induced blastema. Therefore, it appeared that expression of the mesenchymal marker genes was more intense in the center of the blastema (e.g. Figs. 4F, G); however, cells in the less dense peripheral regions also expressed these genes. Although expression of *AmPrx-1* and *AmTbx-5* was detected by RT-PCR at low levels in the uninjured skin (Fig. 6A), we did not detect expression by *in situ* hybridization in either the mature skin or in wounds without a deviated nerve (Figs. 3N, O).

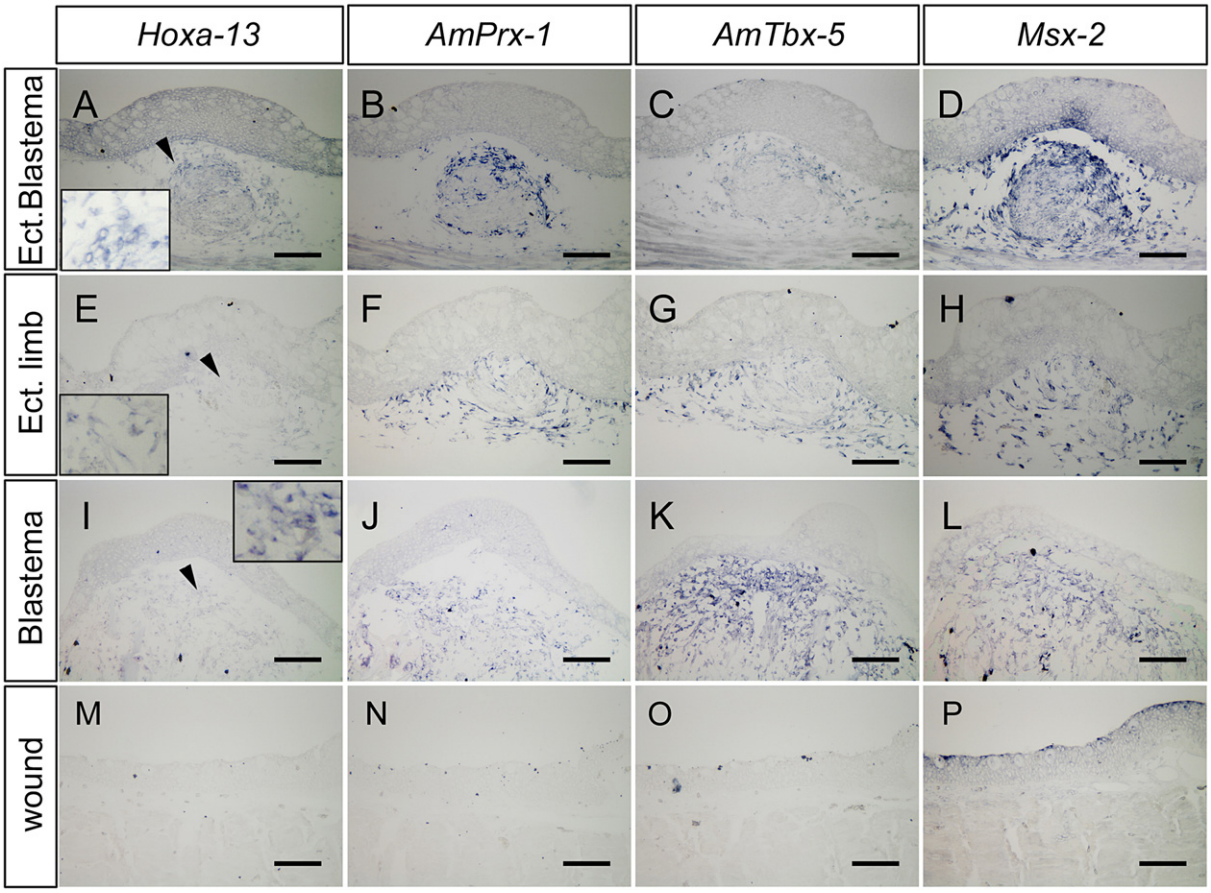

Fig. 3. Comparison of the patterns of gene expression at early stages of regeneration in ectopic blastemas (A–D; Ect. Blastema) that will not form a limb (no skin graft), ectopic blastemas with a skin graft that will form a limb (E–H; Ect. Limb), amputation-induced blastemas (I–L) and a simple wound without a deviated nerve (M–P). Expression of four early regeneration marker genes is illustrated: *Hoxa-13* (A, E, I, M); *AmPrx-1* (B, F, J, N); *AmTbx-5* (C, G, K, O); and *Msx-2* (D, H, L, P). *Hoxa-13* was expressed at low level in all three types of blastemas at this early stage of regeneration (A, E, I), but not in simple skin wounds (M). Inserts in panels A, E, and I are higher magnification views of the regions indicated by arrowheads. Scale bars = 200 μm.

As in amputated limbs, *Mmp-9* was one of the earliest genes to be expressed in lateral wounds, regardless of whether or not they had received a nerve or skin graft. Expression was predominantly localized to the basal keratinocytes of the wound epidermis (Figs. 5F, G), though there was expression in some of the underlying mesenchymal cells. As noted above, *Mmp-9* was expressed in the wound epidermis of a simple wound but was rapidly downregulated and was not detected at 24 h post-wounding (Fig. 5E). In contrast, expression was maintained for several days in response to a deviated nerve both with and without a contralateral skin graft as well as in amputated limbs in the same patterns as observed at 24 h (Figs. 5F, G).

Along with *Mmp-9*, *Msx-2* was one of the earliest up-regulated genes in response to wounding and nerve deviation. As reported above, *Msx-2* was detected weakly in epidermal cells of intact limbs using our *in situ* hybridization protocol (data not shown). This low level expression was upregulated in the wound epidermis and underlying mesenchymal cells of a simple wound after 24 h (Fig. 5A) but was no longer detected at high levels after 48 to 72 h in wounds without a deviated nerve (data not shown). In contrast, *Msx-2* continued to be expressed at an elevated level in the same patterns as observed at 24 h (Figs. 5B–D) in response to signals from a deviated nerve whether or not there was also a contralateral skin graft. This sustained expression was comparable to what was observed in response to amputation. As with *AmPrx-1* and *AmTbx-5*, *Msx-2* was expressed by all mesenchymal cells within ectopic blastemas and ectopic limbs (Figs. 3D, H; Figs. 4D, H). In progressively later blastema stages, expression of *Msx-2* in mesenchymal cells increased, whereas expression in the blastema epithelial cells decreased. *Msx-2* expression was detected by RT-PCR at low levels in uninjured skin (Fig. 6A) and was also observed by *in situ* hybridization in wounds without a deviated nerve (Fig. 3P).

Hoxa-13 was not detected by RT-PCR in uninjured skin (Fig. 6A) or in wounds without a deviated nerve, whether or not there was a contralateral skin graft (Fig. 6B). Consistent with these data, we did not observe *Hoxa-13* expression by *in situ* hybridization in wounds without a deviated nerve (Fig. 3M). In contrast, a deviated nerve induced *Hoxa-13* expression (Figs. 3A, E) in a fashion that was comparable to what was observed in response to limb amputation (Fig. 3I). During the early stages of ectopic blastema formation, *Hoxa-13* was expressed by all mesenchymal cells (Figs. 3A, E), but at later stages, expression became more distally restricted (Figs. 4A, E). *Hoxa-13*

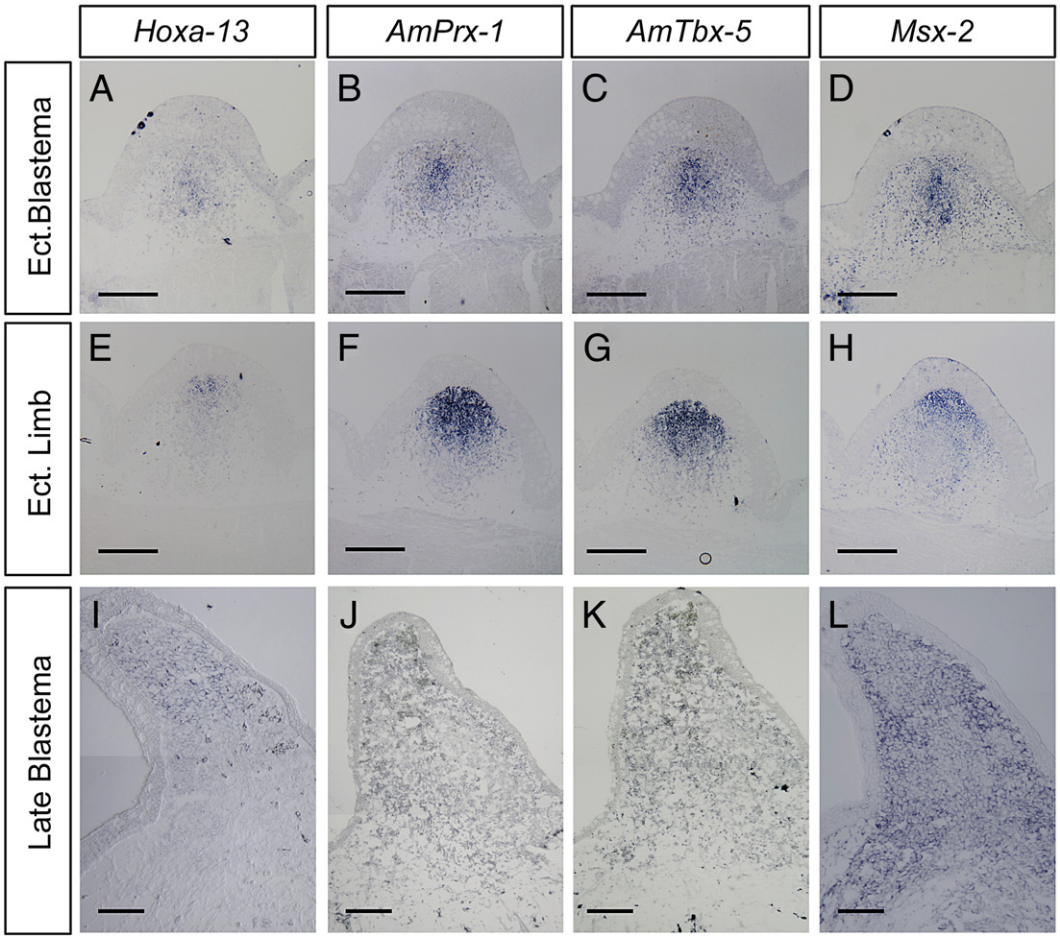

Fig. 4. Comparison of the patterns of gene expression at later stage of regeneration relative to Fig. 3, in ectopic blastemas (A–D; Ect. Blastema) that will not form a limb (no skin graft), ectopic blastemas with a skin graft that will form a limb (E–H; Ect. Limb), and amputation-induced blastemas (I–L). Expression patterns of the same regeneration marker genes as in Fig. 3 are illustrated: *Hoxa-13* (A, E, I); *AmPrx-1* (B, F, J); *AmTbx-5* (C, G, K); and *Msx-2* (D, H, L). *AmPrx-1*, *AmTbx-5*, and *Msx-2* were expressed throughout the blastema mesenchyme, whereas *Hoxa-13* was not expressed in proximal regions (A, E, I). Scale bars in panels A–H = 500 μm. Scale bars in panels I–L = 200 μm.

expression was not detected in the epidermis at any stage of blastema development.

To confirm the gene expression patterns observed by *in situ* hybridization, we performed RT-PCR (Fig. 6). RT-PCR revealed that the uninjured, mature skin expressed *AmTbx-5* and *Msx-2* weakly, and *AmPrx-1* at a higher level. *Hoxa-13* was not expressed in uninjured skin. Expression of all four genes was either induced (*Hoxa-13*) or upregulated in ectopic blastemas and limbs, as well as in amputation-induced blastemas (Fig. 6A). Although the induction of *Hoxa-13* expression by a deviated nerve did not require a contralateral skin graft, a contralateral skin graft without a deviated nerve was not sufficient to induce ectopic *Hoxa-13* expression (Fig. 6B). We grafted posterior skin to an anterior wound without also deviating a nerve and assayed for the expression of *Hoxa-13* 5 days after surgery. Expression of *Hoxa-13* was not detected in wounds without a deviated nerve whether or not they had received a contralateral skin graft (Fig. 6B), which is consistent with the finding that contralateral skin grafts without a deviated nerve are not sufficient to induce formation of an ectopic blastema (Endo et al., 2004).

Ectopic blastema cells interact with amputation-induced blastema cells and participate in regeneration of an amputated limb

As an additional test of the hypothesis that ectopic blastema cells are equivalent to amputation-induced blastema cells, we determined whether these two cell types could interact in a coordinated fashion to make a new limb. Previous studies had demonstrated that mesenchymal cells from an amputation-induced blastema that had been dissociated and grafted back into a host blastema could participate in regeneration (Groell et al., 1993). We extended this observation by analyzing the contribution and fate of the grafted blastema cells (Figs. 7A–F). Immediately after surgery, the grafted cells were localized beneath the host blastema epidermis (Figs. 7A, B). As the blastema grew, grafted cells moved distally and some maintained their distal location relative to the tip of the regenerating host blastema (Figs. 7C, D). By day 20, digits were forming, and grafted cells were observed along the proximal–distal axis of the regenerated limb, including in the most distal, autopod region (Figs. 7E, F). We observed the same result when cells

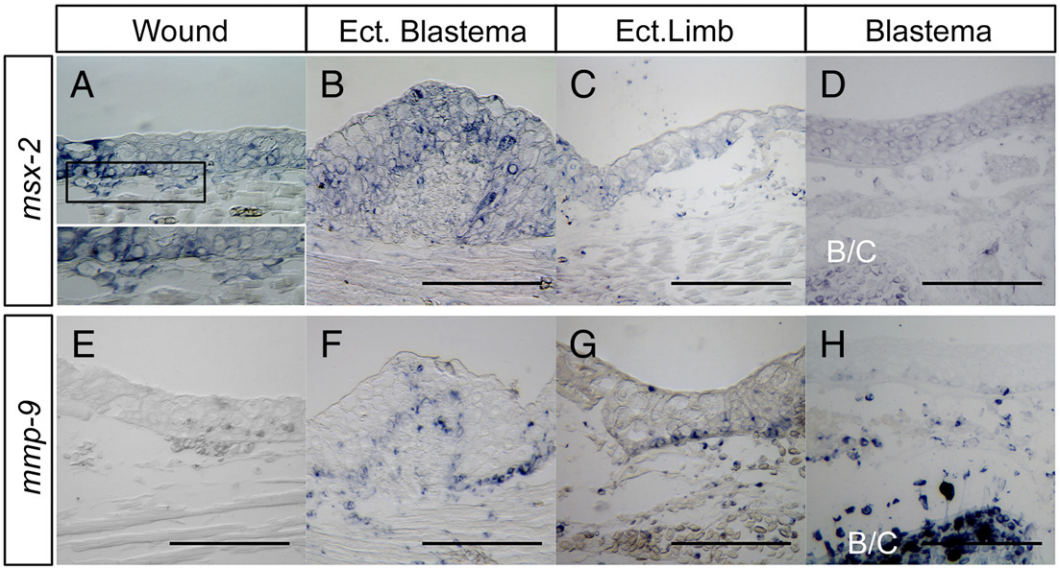

Fig. 5. Expression of *Msx-2* and *Mmp-9* in response to injury. Samples were analyzed 24 h after a simple skin wound (A, E), a wound with a deviated nerve but no skin graft (B, F; Ect. Blastema), a wound with a deviated nerve and a contralateral skin graft (C, G; Ect. Limb), and a wound created by a limb amputation (D, H; Blastema). Although simple wounding did not give rise to a blastema, *Msx-2* expression was still evident in both ectoderm and underlying mesenchyme after 24 h (A, the insert at the bottom of the image is a high magnification view of the area indicated by the box in panel A) but was downregulated within another day (data not shown). In the three types of wounds that formed a blastema (B–D), *Msx-2* expression persisted. *Mmp-9* expression was induced rapidly in response to wounding but was not detected 24 h later in simple skin wounds (E). *Mmp-9* expression was still elevated at 24 h in the three types of wounds that formed blastemas (F–H). *Mmp-9* was expressed at high levels in the amputated distal tip of the bone and cartilage (B/C in panel H). The amputation-induced blastema wound epidermis (D, H) is visualized in a longitudinal section of the amputated limb oriented such that dorsal is to the left and distal (amputation plane) is up. Scale bars = 200 μm.

from ectopic blastemas were similarly grafted into an amputation-induced blastema (Figs. 7G–L). Therefore, ectopic blastema cells behave the same as amputation-induced blastema cells when grafted into a regeneration-permissive environment (a medium bud blastema formed at the site of an amputation).

We investigated the pattern of contribution of grafted ectopic blastema cells to the final regenerate in tissue sections (Fig. 8). Consistent with the whole-mount observations, grafted ectopic blastema cells contributed to regenerated structures at all levels along the proximal–distal axis (Fig. 8C), including the most distal tip (Fig. 8E). In addition to contributing to the regenerated connective tissues, the grafted cells contributed extensively to the regenerated cartilage (Fig. 8G) and joints (Fig. 8E).

To determine if the behavior of grafted ectopic blastema cells was related to their ability to participate in regeneration, we tested the behavior of non-limb dermal fibroblasts that are unable to support limb regeneration. If the limb skin is replaced with head skin that is allowed to heal into place prior to amputation, the chimeric limb fails to regenerate when amputated (Effimov, 1931; Tank, 1981, 1983; Thornton, 1962; Trampusch, 1958). We therefore tested the contribution and fate of head skin fibroblasts when grafted into a host blastema (Fig. 9). Grafted head skin fibroblasts survived at the graft site but did not contribute to any of the regenerated structures that formed at levels more distal to the graft site (Fig. 9C). No grafted cells were observed at the distal tip of the regenerate (Fig. 9E). In contrast to grafted ectopic blastema cells, head skin fibroblasts only contributed to the connective tissue at the graft site, and no grafted cells contributed to the regenerated cartilage (Fig. 9G).

As a final test of the hypothesis that ectopic blastema cells are equivalent to amputation-induced blastema cells, we determined if ectopic blastema cells have positional information, and if they can use that information to interact with host blastema cells with the opposite positional information. Positional interactions between graft and host cells from amputation-induced blastemas induce the formation of supernumerary digits when posterior blastema cells are grafted into an anterior site of the host regenerating blastema (Groell et al., 1993). Conversely, interactions between cells with the same positional information (anterior cells grafted into an anterior host site) do not induce supernumerary structures, and the regenerated pattern is normal. To test for anterior and posterior positional information in ectopic blastema cells, we grafted cells from ectopic posterior blastemas to the anterior side of a host blastema, as well as cells from anterior ectopic blastemas to the same anterior blastema host site. Both posterior and anterior ectopic blastema cells survived after grafting. Grafted posterior ectopic blastema cells induced a supernumerary structure in 67% ($n=6$) of the grafts (Fig. 10). These limbs showed bifurcated digits and/or supernumerary digits (Figs. 10B–D). In contrast, none of the anterior ectopic blastema grafts induced the formation of supernumerary structures ($n=7$). Thus, ectopic blastema cells have positional information and utilize that information to interact with amputation-induced blastema cells.

Discussion

The results of the present study provide molecular and functional validation of the Accessory Limb Model (ALM; Endo et al., 2004) for understanding the mechanisms regulating

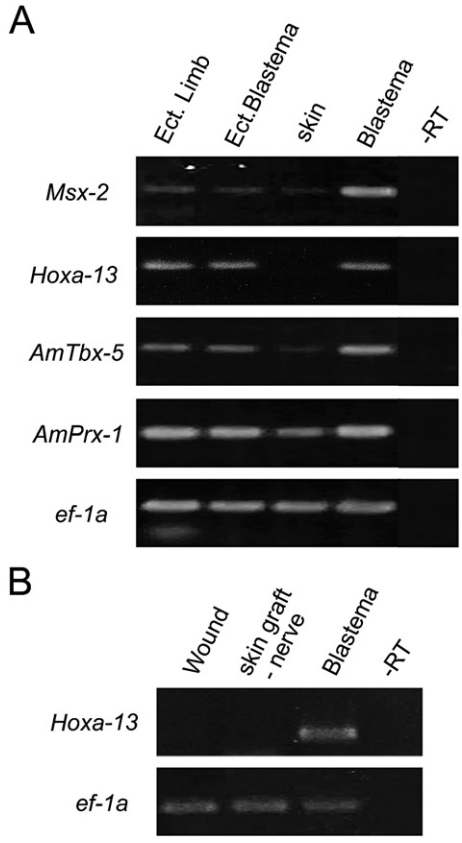

Fig. 6. RT-PCR validation of expression of the four regeneration genes analyzed by *in situ* hybridization as illustrated in Figs. 3 and 4. (A). Expression was analyzed for uninjured limb skin (skin), a medium bud amputation blastema (blastema) and ectopic blastemas collected 10 days after surgery (Ect. Blastema=nerve deviation without a skin graft; Ect. Limb=nerve deviation with a contralateral skin graft). (B) A contralateral skin graft without a deviated nerve is not sufficient to induce ectopic *Hoxa-13* expression (skin graft and no nerve), as is the case of a simple wound (no deviated nerve and no skin graft). In contrast, an amputation-induced blastema does express *Hoxa-13* (blastema).

limb regeneration. Although a lateral wound can be induced to form an ectopic limb in response to a deviated nerve and a contralateral skin graft, it previously was not known whether an ectopic limb was formed using the same molecular mechanisms that regulate regeneration of an amputated limb. In this study, we report that ectopic blastemas and amputation-induced blastemas express key regeneration genes in the same spatio-temporal patterns, that ectopic blastema cells interact with amputation-induced blastema cells to form multiple tissue types in the regenerated limb, and that ectopic blastema cells have position-specific identities corresponding to the origin of their connective tissue progenitor cells. Therefore, the ALM is an appropriate model system for identifying the genetic pathways regulating dermal fibroblast dedifferentiation and blastema formation leading to limb regeneration.

Expression of regeneration marker genes in ectopic blastemas and amputation-induced blastemas is the same

The spatio-temporal expression patterns of the genes analyzed in this study are the same for the three types of outgrowths that can be induced in response to injury; an amputation-induced blastema, an ectopic blastema that will not form a new limb (nerve deviation without a contralateral skin graft), and an ectopic blastema that will form a new limb (nerve deviation with a contralateral skin graft). It is not surprising that ectopic limb blastemas and amputation-induced blastemas are

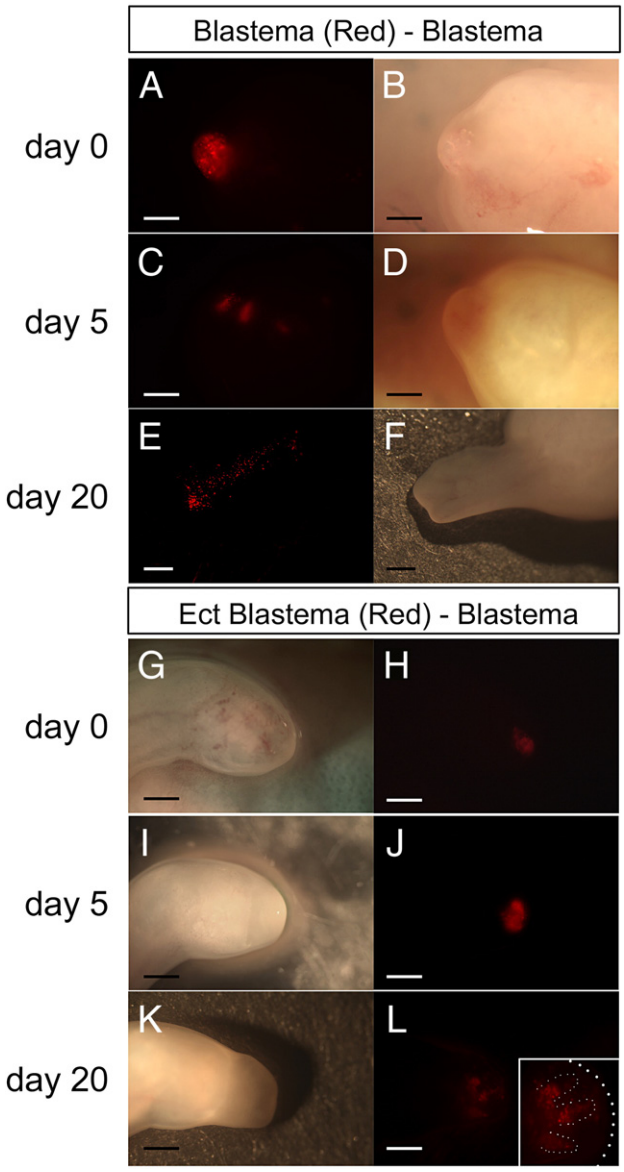

Fig. 7. Grafting of cells from normal blastemas and ectopic blastemas into a normal blastema host. Grafted cells were labeled with red fluorescent dye, and the progression of regeneration was imaged with both bright field optics (B, D, F, and G, I, K) and fluorescence optics (A, C, E and H, J, L). (A–F) Cells from an amputation-induced blastema (normal blastema) were grafted into a host normal blastema as a positive control for normal blastema cell interactions. Grafted cells moved distally (day 5; C, D) and were present at all levels along the proximal–distal axis of the regenerated limb (day 20; E, F). Ectopic blastema cells (no skin graft) participated in regeneration after grafting into a normal host blastema (G–L) in the same fashion as observed for grafts of normal blastema cells (A–F). Insert in panel L is a higher magnification view showing contribution of ectopic blastema cells to the regenerated digits outlined by the small dots. The larger dots indicate the terminal demarcation of the blastema. All limbs are viewed from the dorsal surface with the posterior side oriented toward the top of the image. Scale bars=1 mm.

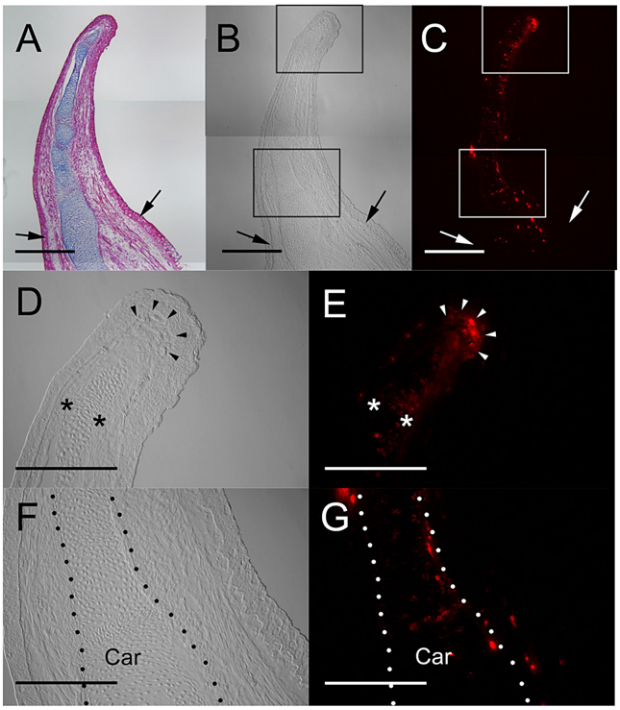

Fig. 8. Analysis in histological section of the contribution of ectopic blastema cells that were grafted into a normal host blastema. (A) Hematoxylin, eosin, and Alcian blue staining. (B) Unstained, bright field view of the section adjacent to (A). (C) Fluorescent view of the section illustrated in panel B. Arrows in panels A–C indicate the cut edge of the thick dermal collagen layer that demarks the original amputation plane at the mid-zeugopod level. Grafted cells were labeled with PKH26 red fluorescent dye. Grafted cells were observed at both proximal and distal levels of the regenerated limb (boxes in panels B and C are corresponding regions of the same section). The images in panels D and E are higher magnification views of the upper boxes in panels B and C. Asterisks in panels D and E indicate the position of a regenerated joint. Arrowheads in panels D and E indicate the distal-most extent of the mesenchyme (basal layer of the distal epidermis). Grafted cells contributed to both the connective tissue and the cartilage (Car) of the regenerated limb (F, G). The regions illustrated in panels F and G correspond to the lower boxes in panels B and C. Longitudinal sections are oriented such that dorsal is left and distal is up for all pictures. Scale bars in panels A–C = 1 mm. Scale bars in panels D–G = 0.5 mm.

similar given that both eventually form identical limb structures. The similarity of gene expression in non-limb forming blastemas (nerve-induced without a contralateral skin graft) indicates that the early events of limb regeneration associated with fibroblast dedifferentiation and blastema formation (blastema morphogenesis) are independent of the later events leading to limb outgrowth and differentiation. Thus, blastema morphogenesis is dependent on a threshold level of innervation (see Wallace, 1981) but is not dependent on the positional interactions required to form an entire limb. We would note that we have only examined genes associated with early events in limb regeneration. Given the differences in regenerative outcome (termination of outgrowth in non-skin grafted ectopic blastemas vs. limb formation in skin grafted ectopic blastemas and amputation-induced blastemas), we assume that the expression of genes that function during the later stages of regeneration is similar for skin-grafted and amputation-induced blastemas, but different for non-skin grafted blastemas.

Although all the genes analyzed are associated with blastema morphogenesis, they are not necessarily expressed in simple wounds that have not been induced to undergo dedifferentiation. It therefore appears that there are signals associated with wounding that induce expression of *Mmp-9* and *Msx-2*, and that these signals are distinct from nerve-derived signals that induce expression of *Hoxa-13*, *AmPrx-1*, and *AmTbx-5*. Since expression of both *Mmp-9* and *Msx-2* is maintained in nerve-deviated wounds, these two genes are either responsive to both wound and nerve-derived signals, or the signals associated with wounds and nerves are the same. In the later case, the wound-associated signals might be derived from subcutaneous nerves, and a higher threshold for induced expression of *Hoxa-13*, *AmPrx-1*, and *AmTbx-5* requires a higher threshold supply of nerves (Peadon and Singer, 1965; Singer, 1945, 1946) that is supplied by the deviated nerve.

Many of the limb regeneration marker genes that we have analyzed have been studied previously and are implicated as having a regulatory function in regeneration. *Mmp-9* is the earliest regeneration marker gene reported to date and is expressed within the first few hours following injury in the migrating wound epidermis (Yang et al., 1999). Inhibition of MMP function inhibits limb regeneration (Vinarsky et al., 2005), and also inhibits reepithelialization (Gardiner, unpublished observations).

Tbx-5 expression has been analyzed previously in the newt, and to a limited degree in the axolotl by immunolocalization with a heterologous antibody (Khan et al., 2002). Consistent with this previous report, we detected axolotl *Tbx-5* mRNA expression in mesenchymal cells of both developing and regenerating forelimbs. This pattern of expression is conserved among all tetrapod limbs examined. Thus, it appears that axolotl *Tbx-5* has a similar role in both limb development and limb regeneration in the specification of forelimb identity.

Expression of *Hoxa-13* is induced in all three types of regeneration-associated outgrowths. As in the case of amputation-induced blastemas, *Hoxa-13* is induced early in association with the mesenchymal cells at the wound site and subsequently becomes distally restricted after the blastema has formed (Gardiner et al., 1995). This spatio-temporal pattern of expression indicates that the distal tip of the pattern is specified as an early event in regeneration. Interaction between the newly respecified distal cells and the more proximal stump tissues stimulates growth and the intercalation of cells with intermediate positional values so as to restore the missing pattern along the proximal–distal axis (Gardiner et al., 1995). This conserved pattern of expression in both types of ectopic blastemas (with and without a contralateral skin graft) indicates that the proximal–distal axis has been respecified in these outgrowths. Again, it is not surprising that ectopic limb blastemas (with a contralateral skin graft) and amputation-induced blastemas exhibit identical patterns of *Hoxa-13* expression given that both eventually form identical limb structures. However, the similarity of *Hoxa-13* expression in non-limb forming blastemas (nerve-induced without a contralateral skin graft) indicates that the early respecification of the proximal–distal axis during blastema morphogenesis is not

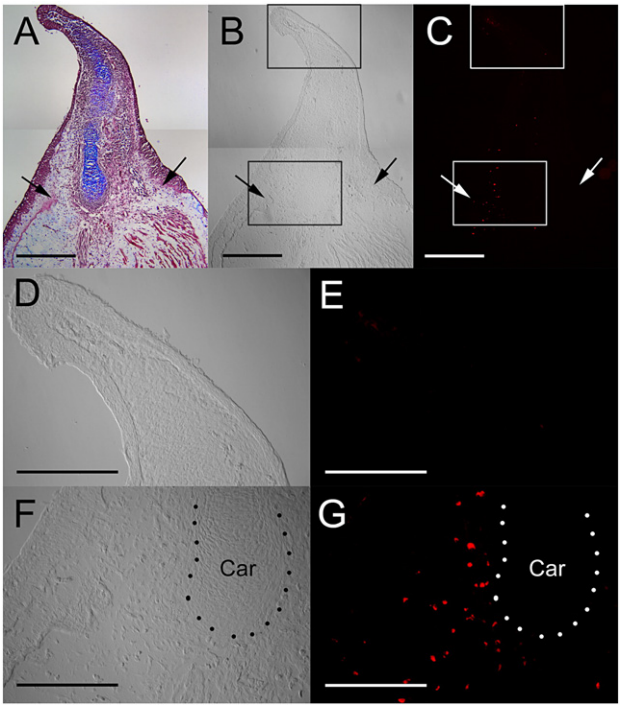

Fig. 9. Analysis in histological section of the contribution of head skin dermal cells that were grafted into a normal host blastema. (A) Hematoxylin, eosin, and Alcian blue staining. (B) Unstained, bright field view of the section adjacent to (A). (C) Fluorescent view of the section illustrated in panel B. Arrows in panels A–C indicate the cut edge of the thick dermal collagen layer that demarks the original amputation plane at the mid-zeugopod level. Grafted cells were labeled with PKH26 red fluorescent dye. Grafted cells were observed at the proximal site of grafting (lower boxes in panels B and C), but not at more distal levels of the regenerated limb (upper boxes in panels B and C). The images in panels D and E are higher magnification views of the upper boxes in panels B and C and demonstrate that no grafted cells contributed to distal structures. Grafted cells contributed to the proximal connective tissue at the site of grafting, but not to the cartilage (F, G). The regions illustrated in panels F and G correspond to the lower boxes in panels B and C. Car=cartilage. Longitudinal sections are oriented such that dorsal is right and distal is up for all pictures Scale bars in panels A–C=1 mm. Scale bars in panels D–G=0.5 mm.

dependent on the interactions between fibroblast-derived cells from opposite positions around the circumference (posterior skin grafted to an anterior host site).

Although contralateral cell–cell interactions are not required for blastema morphogenesis, it is likely that fibroblast interactions are involved in the stimulation of growth as the blastema forms. In an amputated limb, the migration of dermal fibroblasts from around the limb circumference to the center of the wound precedes and likely triggers the onset of proliferation (Gardiner et al., 1986). Similarly, dermal fibroblasts that originate at the periphery of a lateral wound with a deviated nerve are recruited into the center of the wound and contribute to formation of an ectopic blastema (Endo et al., 2004). Since these cells originate from different positions around the wound margin, it is likely that they have different positional identities. Thus, interactions between these cells as they migrate toward the center of the wound would likely stimulate proliferation in a manner comparable to an amputated limb. However, in an amputation wound, cells from all positions around the limb circumference are present; whereas in a lateral wound, the ectopic blastema progenitor cells are only derived from the connective tissues localized to the wound site. Thus, these interacting cells cannot provide the degree of positional confrontation that occurs between cells derived from the opposite side of the limb with either a limb amputation or a contralateral skin graft. Previous studies have repeatedly demonstrated that interactions between cells derived from positions that are greater than one half of the limb circumference apart are required to induce formation of a new limb (see, Bryant et al., 1981; Endo et al., 2004; Maden and Holder, 1984). The mechanisms regulating the later steps that are dependent upon the circumferential positional interactions required for ectopic limb formation are unknown at the present time.

Msx genes are the only genes for which there is direct genetic evidence of a key regulatory function in limb/digit regeneration (Han et al., 2003). Mouse limbs, as well as the limbs of other mammals including humans, have the ability to regenerate the terminal phalanx of digits. The domain of regeneration competence corresponds to the domain of *Msx-1* expression, and digit regeneration in *Msx-1*$^{-/-}$ mice is inhibited. The regeneration defect can be rescued by BMP-4, indicating that *Msx-1* function as a transcriptional repressor is mediated by BMP signaling during blastema morphogenesis (Han et al., 2003). Expression of *Msx* genes has been characterized during blastema morphogenesis in regenerating urodele limbs (Carlson et al., 1998; Koshiba et al., 1998; Simon et al., 1995, and this study). Work in progress to inhibit *Msx-2* function indicates that it has an equivalent function in the regulation of urodele limb regeneration as does *Msx-1* in mouse digit tip regeneration.

In contrast to the other four regeneration marker genes, *Prx-1* expression has not been analyzed previously in either developing or regenerating urodele limbs. *Prx-1* has been used as a limb-specific mesenchymal marker gene in studies of tetrapod limb development (Nohno et al., 1993), and the *Prx-1* promoter has been used to drive transgene expression specifically to the limb bud mesenchyme (Logan et al., 2002; Suzuki et al., 2007, 2006). *AmPrx-1* is highly conserved at the nucleotide sequence level as compared to other tetrapods and is expressed specifically by mesenchymal cells in the developing limb bud. *AmPrx-1* expression and presumably its function are conserved in all three types of blastemas that we examined. It therefore appears that *Prx-1* functions in fibroblast dedifferentiation and early blastema formation, and thus it is a specific marker for blastema morphogenesis.

As part of the present study, we validated our *in situ* hybridization (ISH) results by RT-PCR. In contrast to the ISH results, we detected low level expression of *Msx-2*, *AmTbx-5*, and *AmPrx-1* in uninjured skin. We assume that these differences in detection of gene expression reflect the higher level of sensitivity of RT-PCR as compared to ISH. The functional significance, if any, of low level expression of *AmTbx-5* and *AmPrx-1* in uninjured limb skin is presently unknown. *Msx-2* is expressed in uninjured skin in the axolotl (data not shown), as well as in humans (Stelnicki et al., 1997). Given that expression of *Msx-2* in uninjured skin is observed in both regenerating and non-regenerating animals, it is unlikely to

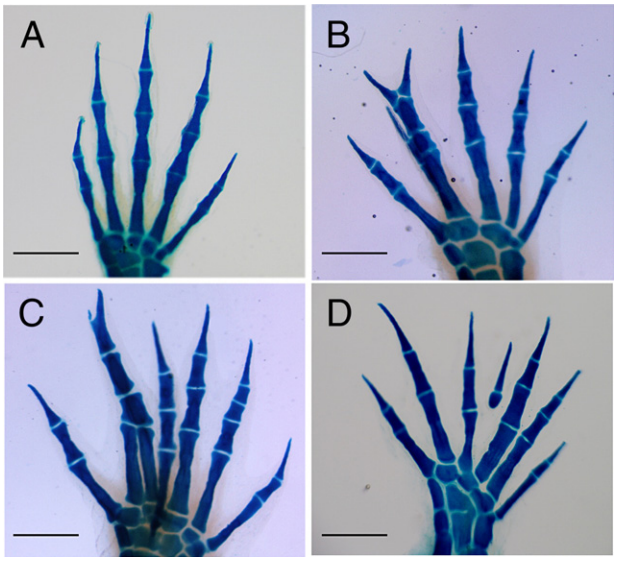

Fig. 10. Position-specific induction of supernumerary digits by ectopic blastema cells. Cells from ectopic blastemas (without a contralateral skin graft) induced on either the posterior or the anterior side of the forelimb were grafted into an anterior host site of a hindlimb blastema induced by limb amputation. Ectopic blastema cells were grafted 10 days after wounding and nerve deviation. (A) The pattern of a hindlimb that regenerated normally after receiving a graft of anterior ectopic blastema cells. (B–D) Examples of supernumerary digits induced by grafts of posterior ectopic blastema cells. All supernumerary digits were induced on the anterior side of the host limb (the limbs are oriented with anterior on the left). Scale bar = 3 mm.

be functionally related to the ability of urodele limbs to regenerate. Finally, since *Hoxa-13* expression was not detected in uninjured skin or in simple wounds without a deviated nerve by RT-PCR, induction of *Hoxa-13* expression is a specific marker for blastema morphogenesis, and presumably is required for successful limb regeneration.

Cells from ectopic blastemas and amputation-induced blastemas interact and have equivalent functions

As discussed above, ectopic blastemas and amputation-induced blastemas are equivalent in terms of gene expression, which implies that they utilize the same molecular mechanisms for blastema morphogenesis. In the present study, we have developed an assay to test whether ectopic blastema cells and amputation-induced blastema cells are functionally equivalent. By following the fate of ectopic blastema cells that have been grafted into an amputation-induced blastema, we have determined that both types of blastema cells are equivalent in terms of migration, proliferation and the ability to interact to regenerate the missing limb structures. This novel assay for the ability of cells to participate in regeneration will allow for the discovery of mechanisms to induce fibroblast dedifferentiation and blastema cells formation.

Although limb dermal fibroblasts can be induced to become limb blastema cells, it appears that non-limb skin fibroblasts cannot. When grafted into a host blastema, head-skin fibroblasts do not migrate distally or interact with the limb blastema cells to form new limb tissues. Although the grafted cells survive, they remain localized at the site of grafting and contribute only to the connective tissues. The non-participation of head skin fibroblasts in the present study is consistent with previous studies demonstrating that limb regeneration is inhibited by a graft of head skin to replace the limb skin (Tank, 1987; Wigmore and Holder, 1986). Regenerative failure in such limbs was hypothesized to be a consequence of the failure head skin fibroblasts to participate in regeneration. Our data are consistent with that hypothesis, and indicate that participation by limb dermal fibroblasts is a consequence of active rather than passive processes. If the translocation of grafted cells to more distal positions were a consequence of being passively displaced or carried along by neighboring cells, then head skin fibroblasts would also be transported distally, which they are not. It thus is likely that distal translocation involves active processes such as migration and intercalation-driven proliferation that are mediated by positional interactions between ectopic limb blastema cells and amputation-induced blastema cells.

It is unclear whether head skin fibroblasts are induced to undergo dedifferentiation after grafting since they had not been induced to form an ectopic blastema prior to grafting, as was the case with grafted ectopic limb blastema cells. Experiments are in progress to determine the sequence of events leading from differentiated dermal fibroblasts to dedifferentiated, functional blastema cells. If the regeneration-permissive environment of the limb blastema is able to induce dermal fibroblasts to dedifferentiate, then presumably head skin fibroblasts dedifferentiate in the host blastema, but retain their identity as non-limb cells and are unable to interact with blastema cells that have a limb identity. Different positional identities of fibroblasts from different tissues and organs, as well as from different positions within the limb, have been characterized at both the cellular and molecular levels (Bryant et al., 1981; French et al., 1976; Rinn et al., 2006). The discovery that not all skin fibroblasts can participate in limb regeneration is of importance in developing strategies to induce human limb regeneration.

Ectopic blastema cells not only participate by contributing cells to the regenerated limb, but also interact with the host blastema cells to control growth and pattern formation. Our previous studies demonstrated that a graft of skin from the opposite side of the limb provided sufficient positional information to form a limb *de novo* (Endo et al., 2004). In the present study, we have discovered that although ectopic blastemas without a contralateral skin graft lack cells with sufficient diversity of positional information to form a limb, the cells nevertheless do have circumferential positional information. The presence of circumferential positional information is revealed by an assay that is based on the well-characterized response to interactions between cells with the same positional identity (no supernumerary structures are induced) in contrast to interactions between cells with different positional identities that stimulation growth and supernumerary pattern formation (Bryant et al., 1981; French et al., 1976). Thus, ectopic blastemas that form on the posterior side of the limb contain blastema cells with posterior positional information, and they induce supernumerary digits when grafted into the anterior side of a host blastema. In contrast, anterior ectopic blastema cells

with anterior positional information are unable to induce supernumerary digits when grafted into the same host site.

Finally, the findings of the present study not only validate the utility of the Accessory Limb Model for discovering the mechanisms of limb regeneration but also demonstrate the feasibility of engineering a functional blastema from limb dermal fibroblasts. It is now evident that in the presence of a wound epidermis and a deviated nerve, limb dermal fibroblasts are induced to undergo dedifferentiation to form a blastema that is equivalent to an amputation-induced blastema. Since an amputation-induced blastema can give rise to a limb when grafted ectopically (Stocum, 1968), our findings are encouraging for the possibility of inducing an equivalent regenerative response in human limbs.

Acknowledgments

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