Hedgehog signaling controls dorsoventral patterning, blastema cell proliferation and cartilage induction during axolotl tail regeneration

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Summary

Tail regeneration in urodeles requires the coordinated growth and patterning of the regenerating tissues types, including the spinal cord, cartilage and muscle. The dorsoventral (DV) orientation of the spinal cord at the amputation plane determines the DV patterning of the regenerating spinal cord as well as the patterning of surrounding tissues such as cartilage. We investigated this phenomenon on a molecular level. Both the mature and regenerating axolotl spinal cord express molecular markers of DV progenitor cell domains found during embryonic neural tube development, including *Pax6*, *Pax7* and *Msx1*. Furthermore, the expression of *Sonic hedgehog* (*Shh*) is localized to the ventral floor plate domain in both mature and regenerating spinal cord. *Patched1* receptor expression indicated that hedgehog signaling occurs not only within the spinal cord but is also transmitted to the surrounding blastema. Cyclopamine treatment revealed that hedgehog signaling is not only required for DV patterning of the regenerating spinal cord but also had profound effects on the regeneration of surrounding, mesodermal tissues. Proliferation of tail blastema cells was severely impaired, resulting in an overall cessation of tail regeneration, and blastema cells no longer expressed the early cartilage marker *Sox9*. Spinal cord removal experiments revealed that hedgehog signaling, while required for blastema growth is not sufficient for tail regeneration in the absence of the spinal cord. By contrast to the cyclopamine effect on tail regeneration, cyclopamine-treated regenerating limbs achieve a normal length and contain cartilage. This study represents the first molecular localization of DV patterning information in mature tissue that controls regeneration. Interestingly, although tail regeneration does not occur through the formation of somites, the Shh-dependent pathways that control embryonic somite patterning and proliferation may be utilized within the blastema, albeit with a different topography to mediate growth and patterning of tail tissues during regeneration.

Key words: Axolotl, Regeneration, Sonic hedgehog, Cyclopamine, Blastema, Sox9, Pax7

Introduction

Axolotl tail regeneration involves the regrowth and patterning of multiple tissue types, including the spinal cord, muscle, cartilage, dermis, fin and skin. After tail amputation and epithelial wound healing, the spinal cord grows out as a tube of neuroepithelial progenitor cells, called the ependymal tube. The regenerating spinal cord is surrounded by blastema cells, the progenitor cells that give rise to the mesodermal tissues in the tail, such as dermis, cartilage and muscle. How coordinated growth and patterning of the different tissue types occurs on a molecular level during axolotl tail regeneration is still unknown. Spinal cord transplantation experiments have established that the spinal cord harbors crucial dorsoventral (DV) patterning information, not only for the regenerating spinal cord but also for the surrounding tissues such as muscle and cartilage (Holtzer, 1956). Holtzer rotated a piece of tail spinal cord 180° about its DV axis, implanted it back into the mature tail and amputated the tail through the operated region. In this situation both the spinal cord and the surrounding tissue regenerated upside down, with cartilage forming dorsally with respect to the whole animal, but still next to the original ventral side of the spinal cord (Holtzer, 1956). This and further work by Holtzer strongly suggests that cartilage is induced by the ventral half of the spinal cord during urodele tail regeneration. It also implies that the mature spinal cord maintains DV patterning information and that this patterning information is transmitted into the regenerate. Similarly, studies in limb regeneration indicate the presence of DV patterning information within the mature limb that is required for proper growth and patterning of the regenerate (Carlson, 1974; Carlson, 1975; Holder et al., 1980).

While the molecular mechanisms underlying spinal cord regeneration are poorly understood, the patterning of the developing neural tube into distinct DV progenitor domains has been molecularly characterized in recent years (reviewed by Bronner-Fraser and Fraser, 1997; Ericson et al., 1997a; Tanabe and Jessell, 1996). The neural tube is subdivided into distinct domains, as defined by a series of homeodomain and paired box-containing transcription factors, with the dorsalmost domain defined by *Msx1* and *2* expression, dorsolateral cells by *Pax7*, and lateral domains by *Pax6*, while *Nkx6.1* and *Nkx2.2* define increasingly ventral domains. The size and placement of these domains is controlled by several morphogens. Sonic hedgehog (Shh), a cholesterol-modified

extracellular signaling factor expressed in the notochord and the floor plate, induces ventral neural tube cell types in a concentration-dependent manner (Briscoe et al., 1999; Ericson et al., 1997a; Ericson et al., 1997b; Litingtung and Chiang, 2000; Roelink et al., 1995). The Shh gradient in the neural tube is antagonized by dorsally secreted bone morphogenetic proteins (Bmps) from the epidermal ectoderm and the dorsal roof plate cells of the neural tube (Liem et al., 1995), which specify a subset of interneurons in the dorsal neural tube (Lee et al., 2000; Liem et al., 1997). Whereas Bmp4 and Bmp7 activate the expression of *Msx1*, *Pax7* and *Pax6* in the dorsal and lateral neural tube, Shh has a concentration-dependent inhibitory effect on the expression of these markers (Goulding et al., 1993; Liem et al., 1995; Timmer et al., 2002). Low concentrations of Shh block *Msx1* and *Pax7* expression but can elevate *Pax6* expression in lateral neural tube cells. High concentrations of Shh, however, inhibit *Pax6* expression in floor plate cells of the neural tube (Ericson et al., 1997b). Thus, during embryogenesis, the notochord ventrally and the ectoderm dorsally impose DV patterning on the neural tube through extracellular signaling.

During development, the action of Shh and Bmps is not restricted to patterning the neural tube. These morphogens also play important roles in controlling cell proliferation, patterning and cell-type specification of somite-derived cells such as the sclerotome, resulting in a coordinated patterning of the neural tube and its surrounding mesodermal structures. *Shh* mutant mice lack vertebral columns and ribs, demonstrating that Shh signaling from the notochord and ventral neural tube is crucial for sclerotome development (Chiang et al., 1996). More specifically, Shh induces the expression of sclerotomal markers such as *Pax1* and *Sox9* (Fan and Tessier-Lavigne, 1994; Marcelle et al., 1999; Murtaugh et al., 1999; Zeng et al., 2002), which are essential for sclerotome development and cartilage formation (Bi et al., 1999; Peters et al., 1999). Similarly, Shh regulates myogenic precursors by positively regulating *Myf5* expression (Gustafsson et al., 2002). In addition to sclerotomal and myogenic markers, Shh induces proliferation of the somitic mesoderm (Fan et al., 1995; Marcelle et al., 1999). Shh also negatively regulates its own signaling by upregulation of its own binding receptor *Patched1* (Goodrich et al., 1996). Taken as a whole, this information indicates that Shh signaling plays diverse roles in the somite, namely proliferation, patterning and negative feedback. The interplay of all three may help define the shape and size of the developing sclerotome-derived skeletal components.

We wanted to investigate the molecular identity of the DV patterning information in the axolotl spinal cord, and how the spinal cord communicates it to the regenerating spinal cord, and subsequently to the surrounding blastema tissue. In order to identify the molecular basis of the DV patterning information in the axolotl spinal cord, we asked whether these well-described markers were present in the mature and/or regenerating spinal cord. Here we demonstrate that *Shh*, *Pax6*, *Pax7* and *Msx1* are expressed in their respective domains in the mature axolotl spinal cord as well as in the ependymal tube. This represents the first time that the molecular basis of DV patterning information in the mature axolotl tissue has been defined. *Patched1* expression further indicates that hedgehog signaling occurs both within the spinal cord, and in surrounding blastema cells. By blocking hedgehog signaling through the drug cyclopamine, we show that it is required not only for DV patterning of the spinal cord, but also for overall tail regeneration. Specifically, the proliferation of blastema cells and *Sox9* expression in the ventral blastema is dependent on hedgehog signaling. Therefore the induction of cartilage by the spinal cord during tail regeneration is mediated at least in part through hedgehog.

Materials and methods

Axolotl care

Ambystoma mexicanum (axolotls) were bred in our facility, where they were kept at 18°C in Dresden tap water and fed daily with artemia. For all surgery, animals were anesthetized in 0.01% ethyl-p-aminobenzoate (Sigma). The experiments described here were performed on 3-cm-long larval axolotls.

In situ hybridization on axolotl tail cryosections and sequences of probes used

Axolotl tail tissue was fixed in 4% fresh paraformaldehyde (PFA) overnight at 4°C, washed in PBS, equilibrated in 30% sucrose and embedded in tissue-tek (O.C.T. compound, Sakura). Cryosections 16 μm thick were mounted on Superfrost adhesive slides and dried at room temperature (RT) for several hours. The sections were quickly washed in PBS and treated with hybridization denaturation mix (2% SDS, 100 mmol/l DTT in 1× PBS) for 20 minutes at RT. After three washes in PBS/0.1% Tween, the sections were digested with Proteinase K (2-10 μg/ml) for 5 minutes and post-fixed directly afterward with PFA for 10 minutes at RT. Slides were washed in PBS/Tween and incubated at RT for 15 minutes in triethanolamine with 0.25% acetic anhydride. After several washes in PBS/Tween, slides were prehybridized in hybridization buffer (50% formamide, 5× SSC, 5× Denhardts, 750 μg/ml yeast RNA) for 1 hour at 68°C, and then hybridized overnight at 68°C with 500 ng/ml DIG-labeled probe in hybridization solution. Slides were washed twice an hour at 68°C in post-hybridization solution (50% formamide, 2× SSC, 0.1% Tween) and then 3× 10 minutes at RT in maleic acid buffer (100 mmol/l maleic acid pH 7.5, 150 mmol/l NaCl, 0.1% Tween). Sections were blocked in maleic acid buffer plus 10% goat serum for 1 hour at RT and then incubated overnight at 4°C in blocking buffer plus alkaline phosphatase conjugated anti-DIG antibody (diluted 1:2000). Slides were washed 2× 5 minutes in maleic acid buffer and 2× 20 minutes in alkaline phosphatase buffer (100 mmol/l Tris pH 9.5, 50 mmol/l $MgCl_2$, 100 mmol/l NaCl, 0.1% Tween). Each slide was overlaid with filtered NBT-BCIP (Sigma) for 1-2 days at RT. The staining reaction was stopped with PBS/Tween and the slides mounted in 90% glycerol.

Sense and antisense probes for in situ hybridizations were prepared from the axolotl *Shh* sequence (CO786463), *Msx1* sequence (AY525844), *Pax6* sequence (CO784109), *Ptc1* sequence (AY887138) and *Sox9* sequence (AY894689). *Shh* and *Pax6* sequences were derived from EST sequences (Habermann et al., 2004), while the *Msx1*, *Ptc1* and *Sox9* sequences were obtained by RT-PCR from total embryonic RNA using degenerate primers (primer sequences and PCR conditions available upon request).

Pax7 antibody staining on axolotl tail cryosections

Axolotl tails were fixed in 4% fresh paraformaldehyde (PFA) overnight at 4°C, washed in PBS, equilibrated in 30% sucrose and frozen in tissue-tek (O.C.T. compound, Sakura). Cross-sections of the tail 16 μm thick were processed for immunohistochemistry with the anti-Pax7 mAB (Pax7, Developmental Studies Hybridoma Bank, Iowa, USA). A Cy5-labeled secondary antibody (Dianova, Hamburg, Germany, http://www.dianova.com) was used at 1:200 dilution. Nuclear stainings were done with 1 μg/ml of Hoechst. To calculate the percentage of Pax7-positive cells in the blastema, between 704 and 1128 blastema cells were counted in total per regenerate.

Cyclopamine and agonist treatment

Cyclopamine was purchased from Toronto Research Chemicals. Two hedgehog agonists in the same chemical class as described (Frank-Kamenetsky et al., 2002), but with different EC50 values, were obtained from the Curis Corp (http://www.curis.com/), and tested. Both gave identical results with respect to their EC50 concentrations. Hh-Ag1.9 is available from the Curis Corp. Unless indicated otherwise, larval axolotls were exposed to cyclopamine and the hedgehog agonist directly after tail or limb amputation. Cyclopamine-treated axolotls were kept in 20 ml water plus 600 nmol/l cyclopamine (diluted from 5 mmol/l stock solution in ethanol). Agonist-treated axolotls were kept in 20 ml water plus 4, 40, 100, 300 nmol/l agonist (diluted from 400 µmol/l stock solution in DMSO). Control animals were kept in 20 ml water, or 20 ml water plus 0.0125% ethanol, or 20 ml water plus the agonist-equivalent amount of DMSO, or 20 ml water plus 600 nmol/l tomatidine (Toronto Research Chemicals).

Cumulative BrdU labeling and anti-BrdU antibody staining

Axolotl tails were amputated and treated with 600 nmol/l cyclopamine or equivalent amounts of ethanol. Animals were injected intraperitoneally with 10 mg of BrdU (in a volume of 10 ml) every 8 hours starting 3 days post-amputation (dpa). Tails were fixed in 4% freshly made PFA 48 and 72 hours after the initial BrdU injection. Cryosections 16 µm thick were prepared and processed for antibody staining with mouse monoclonal anti-BrdU antibody directly coupled to rhodamine (Tanaka et al., 1997). Nuclear staining was performed using 1 µg/ml Hoechst. The percentages of BrdU-positive ependymal cell nuclei, and BrdU-positive blastema cell nuclei ventral to the ependymal tubes were calculated. The graphs in Fig. 6 represent the mean percentage of BrdU-positive cells of 2-4 regenerates. Between 76 and 255 cells in total were counted in ependymal tube and blastema per regenerate.

Spinal cord removal from the axolotl tail

Axolotls were anesthetized and the tail was sliced open from the dorsal side until the level of the spinal cord. The spinal cord was removed over the length of several segments and the tail was allowed to heal for several days. Mock operated axolotl tails were opened until the level of the spinal cord and allowed to heal without removal of the spinal cord. Amputation was performed a few days after the operation.

Results

Shh, *Pax6*, *Pax7* and *Msx1* are expressed in the mature and regenerating axolotl spinal cord

In order to determine the molecular nature of DV patterning in the axolotl spinal cord, we examined the expression of marker genes that characterize the DV axis of the developing neural tube. We chose the secreted signaling molecule *Shh* and the transcription factors *Pax6*, *Pax7* and *Msx1* as well-described and distinct markers of dorsoventral neural progenitor cell populations in the embryonic neural tube (Echelard et al., 1993; Jostes et al., 1990; Robert et al., 1989; Walther and Gruss, 1991). All these markers were expressed in both the mature axolotl spinal cord and in the ependymal tube in DV domains very similar to those found in development. *Shh* was expressed in the ventralmost cells of the axolotl spinal cord (the floor plate) (Fig. 1A-C); *Pax6* was expressed in the lateral spinal cord cells (Fig. 1D,E); and Pax7 was expressed in the dorsolateral cells of the spinal cord (Fig. 1F,G). In addition to the expression in the spinal cord, Pax7 was also expressed in the lateral edges of the blastema (Fig. 1G). It is likely that these Pax7-positive blastema cells represent muscle progenitor cells, as Pax7 is a known satellite cell marker, and the Pax7-positive cells in the mature tail laid adjacent to muscle fibers (data not shown). *Msx1* was expressed in the dorsalmost spinal cord, the roof plate cells (Fig. 1H-J). In this analysis it was crucial to perform the gene and protein expression analysis on tissue sections rather than whole-mount preparations. With whole mounts, spinal cord staining was observed in the regenerate but not the mature tissue, presumably due to insufficient penetration of in situ probe and antibody in the mature part of the tissue, and thus would have given the false impression of a regeneration-specific upregulation of the genes.

Our gene expression analysis indicates that the putative progenitor cells in the mature axolotl spinal cord show an embryonic pattern of DV neural tube markers. In addition, the same DV pattern is present in the ependymal tube throughout axolotl tail regeneration.

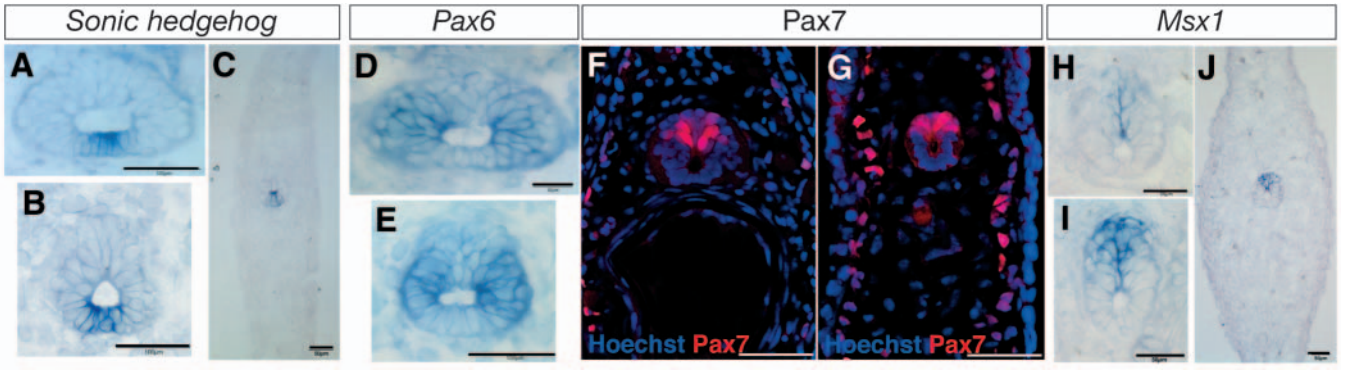

Fig. 1. *Shh*, *Pax6*, Pax7 and *Msx1* are expressed in the differentiated and regenerating axolotl spinal cord. All panels are cross-sections with the dorsal side up. (A-C) *Shh* is expressed in the floor plate of the differentiated spinal cord (A) and in the ventralmost ependymal cells 6 dpa (B,C). The overview in C shows that *Shh* is expressed exclusively in the spinal cord. (D,E) *Pax6* is expressed in the lateral cells of the differentiated axolotl spinal cord (D) and in the lateral ependymal cells 8 dpa (E). (F,G) Pax7 is expressed in the dorsolateral cells of the differentiated spinal cord (F) and in the dorsolateral domain of the ependymal tube 6 dpa. In addition, Pax7 is expressed in lateral tail cells (F,G). (H-J) *Msx1* is expressed in the roof plate of the differentiated spinal cord (H) and in the dorsalmost ependymal cells 4 and 5 dpa (I,J for overview). Panels A-E,H-J show in situ hybridizations, panels F,G antibody staining. Scale bars: 100 µm in A,B,E-G; 50 µm in C,D,H-J.

Hedgehog signaling is required for overall tail regeneration

Shh is a potent morphogen patterning the ventral half of the spinal cord, which leads to the correct spatial organization of interneurons and motoneurons during development (reviewed by Litingtung and Chiang, 2000; Marti and Bovolenta, 2002). Because *Shh* was expressed in the mature and regenerating axolotl spinal cord, we wanted to assess its function in the establishment of the DV identity of the regenerating tail. An interesting question for us was whether interfering with the DV pattern in the regenerating spinal cord would have an effect on the overall DV organization of the regenerating tail: for example, on the position of cartilage formation. Furthermore, we wanted to examine whether Shh is necessary for ependymal cell proliferation, as it has been shown that Shh can act as a mitogen on neural progenitor cells, both in vitro and in vivo (Bambakidis et al., 2003; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005).

In order to inhibit the Shh signaling pathway during tail regeneration, we turned to the widely used chemical inhibitor cyclopamine, which blocks hedgehog signaling by antagonizing the hedgehog receptor Smoothened (Chen et al., 2002; Taipale et al., 2000). The drug can be easily administered through the axolotl water. Interestingly, we found that in the presence of cyclopamine overall axolotl tail regeneration was strongly inhibited (Fig. 2A-G). Wound healing and fin formation occurred normally, and the ependymal tube grew to a limited extent, but a proper blastema did not grow (compare Fig. 2A-C with D-F). The rate of ependymal tube growth was substantially lower than control regenerates (Fig. 2G). In terms of the blastema phenotype, few blastema cells had accumulated in cyclopamine-treated regenerates 4 days post-amputation (dpa) in comparison with the control (compare Fig. 2A with D). The effect became more evident at later stages of regeneration, when even up to 14 dpa neither cartilage nor muscle differentiation took place in cyclopamine-treated regenerates (compare Fig. 2B,C with E,F). Cartilage and muscle started to differentiate at 6 and 10 dpa, respectively, in control regenerates (not shown). After 8 days of cyclopamine treatment, the initial outgrowth of the ependymal tube stopped, and the tube slowly regressed over the following days (Fig. 2G). The inhibitory effect of cyclopamine on tail regeneration could be observed at concentrations ranging from 600 nmol/l to 6 μmol/l, while the same concentrations of tomatidine, a closely related compound to cyclopamine that does not interfere with Shh signaling, did not have this effect (Fig. 2G). As the various concentrations of cyclopamine tested all yielded very similar results, only the lowest concentration (600 nmol/l) was used for the experiments reported here.

Experimental evidence that cyclopamine exerts a specific inhibition of the hedgehog signaling pathway during tail regeneration was the ability to rescue the phenotype with a hedgehog-pathway agonist. When we added a hedgehog agonist in the same chemical class described in Frank-Kamenetsky et al. (Frank-Kamenetsky et al., 2002) (see Materials and methods) together with cyclopamine, a tail with normal cartilage and muscle patterning regenerated (Fig. 2H-K).

The inhibition of tail regeneration by cyclopamine and the rescue of this phenotype with a hedgehog-pathway agonist strongly suggest that hedgehog signaling is required for overall tail regeneration.

Hedgehog signaling is necessary for the correct establishment of DV progenitor domains in the ependymal tube during tail regeneration

When we examined the ependymal tube in cyclopamine-treated regenerates for DV patterning defects we observed expansion of the dorsal spinal cord markers Pax7 and *Msx1* into more ventral regions (compare Fig. 3A,D with Fig. 1G,I). In cyclopamine and agonist-treated regenerates, both the *Msx1* and *Pax7* expression domains were restored, demonstrating the rescue of the cyclopamine effect (compare Fig. 3B,E with Fig. 1G,I). Treatment of regenerating tails with the agonist alone did not have any overall morphological effects, although the regenerating tails might have been slightly bigger. We did, however, observe an effect of the agonist alone on DV patterning markers in the spinal cord. Even low concentrations (4 nmol/l) of agonist abolished Pax7 expression from the dorsal spinal cord (Fig. 3C). By contrast, Pax7-positive cells in the surrounding lateral blastema tissue, which presumably represent muscle progenitors, persisted in the presence of agonist (Fig. 3C).

We conclude from these results that hedgehog signaling is required for the correct establishment of DV progenitor domains in the regenerating axolotl spinal cord.

Patched1 is expressed in the ependymal tube as well as in the blastema

As the strongest effect of inhibiting hedgehog signaling during tail regeneration was a reduced tail blastema, we wanted to know whether blastema cells directly receive the hedgehog signal. We therefore examined the expression of the hedgehog binding receptor *Patched1* (*Ptc1*) in tail regenerates by in situ hybridization. In normal regenerates *Ptc1* was expressed in ventral and lateral spinal cord cells, and in the blastema cells surrounding the ventral spinal cord (Fig. 4B,C; note the absence of staining in the epidermis). *Ptc1* itself is a target gene of the hedgehog signaling pathway that is upregulated where Shh signaling occurs (Goodrich et al., 1996). Agonist-treated regenerating tails showed increased *Ptc1* expression: most or all of the ependymal cells and also most of the blastema cells expressed *Ptc1* (Fig. 4E,F). Together these data indicate that blastema cells receive the hedgehog signal directly. Although it is not known if other hedgehog family members are also expressed during regeneration, the expression of *Ptc1* in the ependymal tube and the surrounding blastema tissue is consistent with the regenerating spinal cord as the primary source of hedgehog signal.

Hedgehog signaling is required for *Sox9* expression in the tail blastema

Knowing that Shh signaling can occur from the ependymal tube to the surrounding blastema cells, we wanted to investigate whether Shh is required for patterning the blastema tissue. During development, Shh induces the expression of the early cartilage marker *Sox9* in the sclerotome (Tavella et al., 2004; Zeng et al., 2002). We examined whether cartilage progenitors in the early blastema express *Sox9*, and whether this expression is controlled by hedgehog signaling. We found that *Sox9* was expressed in a defined area of the blastema ventral to the spinal cord from 4 dpa onward (data not shown), which is 2 days before obvious cartilage differentiation. By contrast, *Sox9* expression was not detectable in cyclopamine-

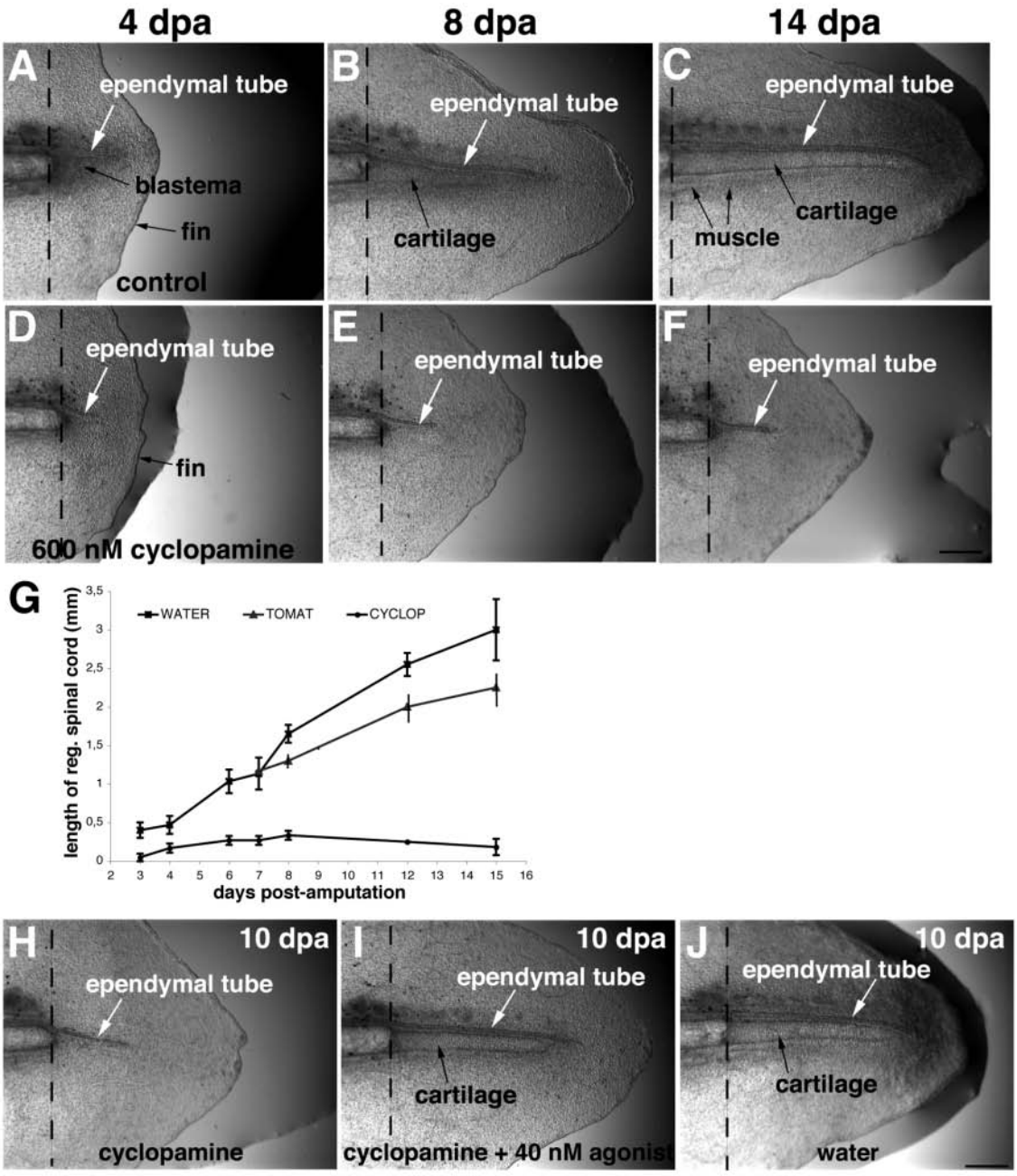

Fig. 2. Cyclopamine treatment blocks axolotl tail regeneration and a hedgehog agonist rescues the phenotype. (A-C) Control regenerates 4, 8 and 14 dpa. The blastema cells are surrounding the ependymal tube at 4 dpa (A). Cartilage has differentiated 8 dpa (B) and muscle 14 dpa (C). (D-F) Cyclopamine-treated tail regenerates 4, 8 and 14 dpa. The fin and the ependymal tube have grown, but a blastema is not visible (compare A and D). No cartilage has differentiated 8 dpa (E) and no muscle has formed 14 dpa (F). (G) Quantification of the lengths of control and cyclopamine-treated tail regenerates over time. The length of the regenerating spinal cord is taken as a measurement for overall tail regeneration. Data points represent the average length of three regenerates; error bars are standard deviations. (H-J) Cyclopamine-treated regenerate (H), cyclopamine and agonist-treated regenerate (I), and normal tail regenerate (J) 10 dpa. The tails in I and J are indistinguishable. (K) Quantification of regenerate lengths (as in G) in indicated conditions shows that 40 nmol/l of the agonist are sufficient to rescue the cyclopamine effect, whereas 4 nmol/l are not. The dashed line in A-F and H-J marks the amputation plane. Scale bars: 0.5 mm.

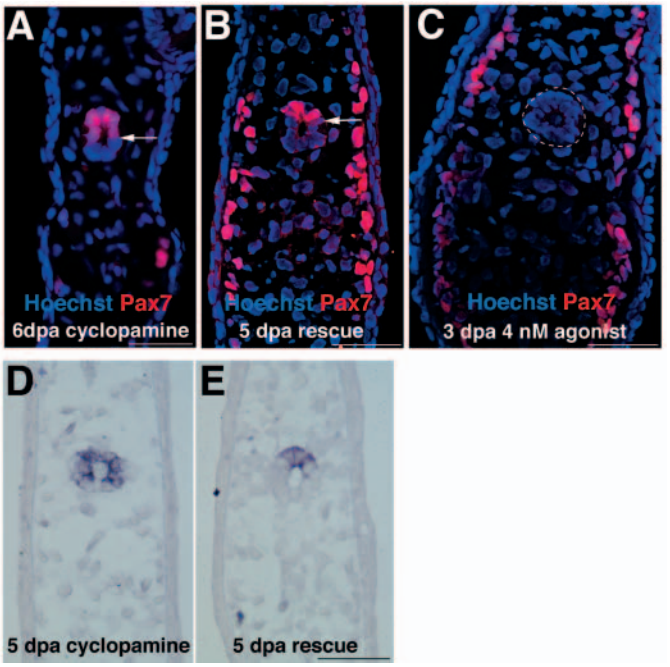

Fig. 3. Dorsal spinal cord progenitor domains are increased in cyclopamine-treated and normal in rescued regenerates. (A-C) Pax7 antibody stainings on cross-sections of cyclopamine-treated regenerate (A), rescued regenerate (B), and agonist- treated regenerate (C). The Pax7 expression domain in the dorsal ependymal tube is expanded ventrally in A, normal in B (compare to Fig. 1G), and absent in C (dashed line in C marks the ependymal tube). The arrows in A,B point to the ventral border of Pax7 expression in the ependymal tube. Note that the Pax7 expression persists in the lateral blastema cells in C. Pax7 staining is in red and nuclear Hoechst staining in blue. (D,E) In situ hybridization of *Msx1* on cross-sections of cyclopamine-treated (D) and rescued regenerates (E). *Msx1* expression in the ependymal tube of cyclopamine-treated regenerates is strongly expanded laterally (D, compare with Fig. 1I). The rescued tails show normal *Msx1* expression (F). Scale bars: 100 μm.

treated regenerates 6 dpa (Fig. 5A,B), while agonist-treated regenerates showed an increased expression domain of *Sox9*, and occasional dorsal blastema cells expressing the gene (Fig. 5C, arrows point to *Sox9*-positive cells). Despite this expanded expression of *Sox9* in the agonist-treated sample, no overt cartilage differentiation was observed in the dorsal blastema, and the ventral cartilage rod appeared normal.

We further examined whether hedgehog signaling is required for the putative muscle progenitors during tail regeneration. It was evident that cyclopamine-treated tail blastemas contained fewer Pax7-positive cells (compare Fig. 3A with Fig. 1G and Fig. 3B). We quantified the percentage of cells in the blastema that were Pax7-positive and found that it was reduced to approximately half the amount in cyclopamine-treated versus control regenerates (10 versus 23%; Fig. 5D). Whereas *Sox9* expression seemed to be completely abolished from the cyclopamine-treated blastema, Pax7 was still expressed, but the number of Pax7-positive blastema cells was significantly reduced.

Hedgehog signaling controls blastema cell proliferation rather than ependymal cell proliferation

The overall morphology of cyclopamine-treated regenerates indicated that the fin was normal, but the size of the blastema was severely reduced (compare Fig. 2A with 2D). On cross-sections we observed that cyclopamine-treated regenerates had a smaller width compared with controls (compare Fig. 3A with Fig. 1G and Fig. 3B, and Fig. 5A with 5B).

We examined whether the reduction of the blastema was due to apoptosis or a block in cell division. TUNEL staining of cyclopamine and control samples were indistinguishable, suggesting that massive apoptosis did not account for the blastema defect (data not shown). To examine cell proliferation, we performed cumulative BrdU labeling for 48 and 72 hours, starting 3 dpa. The percentage of BrdU-positive ependymal cells and BrdU-positive ventral blastema cells was calculated in control and cyclopamine-treated regenerates at 48 hours after the initial injection. We observed a different effect of cyclopamine on proliferation of ependymal cells versus

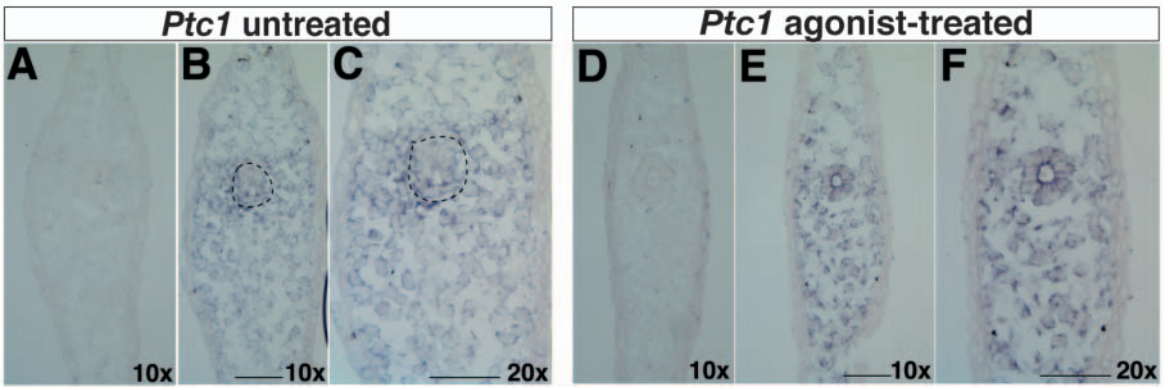

Fig. 4. Ependymal cells and tail blastema cells express the hedgehog receptor *Ptc1*. (A-F) In situ hybridization of *Ptc1* on cross-sections. Sense probe shows no staining of the entire cross-section both in control (A) and agonist-treated (D) regenerates. *Ptc1* is expressed in the ventral ependymal cells (dashed line marks the ependymal tube) and in the blastema, but not in the epidermis (B,C). Note that the staining in the blastema is strongest in the cells surrounding the ventral spinal cord. C is taken at twice the magnification of B. A-C is 4 dpa. *Ptc1* expression is increased in agonist-treated regenerates (E,F). Now all the ependymal cells express *Ptc1* and the vast majority of ventral blastema cells do (compare ventral to dorsal blastema cells). F is taken at twice the magnification of E. D-F is 5 dpa. Scale bars: 100 μm.

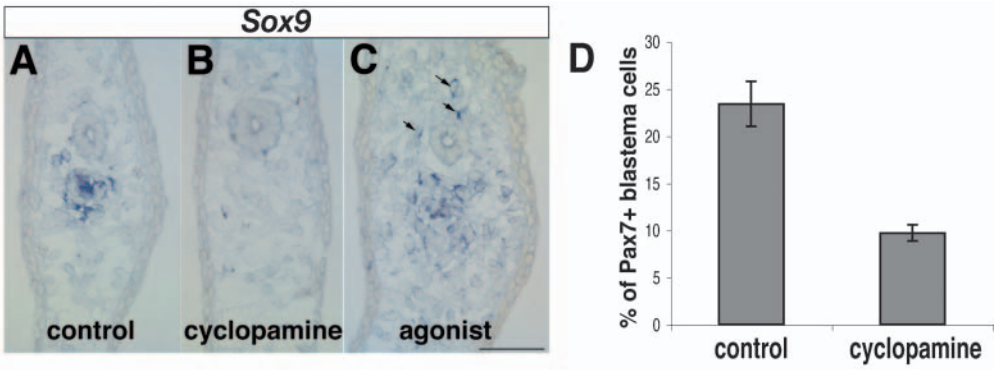

Fig. 5. *Sox9* and Pax7 expression is reduced in cyclopamine-treated blastemas. (A-C) *Sox9* in situ hybridization on cross-sections of control (A), cyclopamine-treated (B) and agonist-treated (C) regenerates 6 dpa. Note the absence of staining in B. The arrows in C point to *Sox9*-positive dorsal blastema cells. (D) Quantification of Pax7-positive blastema cells in control and cyclopamine-treated regenerates 6 dpa. Columns represent the mean percentage of Pax7-positive blastema cells of three regenerates (total number of counted cells per regenerate is between 704 and 1128). Error bars indicate standard deviations. The *t*-test value is 0.0007. Scale bar: 100 μm in A-C.

ventral blastema cells (compare Fig. 6A with 6B). Whereas cyclopamine treatment had only a minor effect on the fraction of proliferating ependymal cells (from 99 to 86%; Fig. 6A), it had a strong, statistically significant inhibitory effect on the fraction of proliferating ventral blastema cells, from 95 to 56% (Fig. 6B). This inhibitory effect was stable over time, as we observed the same decrease of BrdU incorporation at 72 hours, indicating that all proliferating cells had incorporated BrdU. We conclude that hedgehog signaling controls the proliferation of approximately 40% of ventral tail blastema cells. This number could be an underestimate, because we could have inadvertently included some fin cells (that regenerate normally) in the analysis.

Shh has distinct activities on the limb versus the tail blastema

To address whether the cyclopamine effect on tail blastema cell proliferation might represent a nonspecific effect on cell division, we examined whether cyclopamine had a distinct effect on the limb blastema. When we treated regenerating limb blastemas with cyclopamine we obtained limb regenerates of normal length but lacking digits (Fig. 7), consistent with the expected defects in anteroposterior (AP) digit patterning as in the developing limbs of the *Shh* knockout mouse (Chiang et al., 1996), and as previously observed for axolotl limb regeneration (Roy and Gardiner, 2002). We conclude that Shh has different effects on limb versus tail regeneration. Whereas cyclopamine-treated tails showed a profound effect on both growth and patterning of the regenerate, cyclopamine-treated limbs were affected only in the AP patterning of the digits. These data, together with our previous data, further indicate that the block in tail regeneration in response to cyclopamine represents a specific inhibition of the hedgehog signaling pathway.

Ectopic activation of hedgehog signaling in the absence of the spinal cord is not sufficient for tail regeneration

It was previously shown that tail regeneration is absolutely dependent on the presence of the spinal cord at the plane of amputation. Removal of the spinal cord from the distal tip of the tail and subsequent tail amputation blocks growth until the spinal cord regenerates back (Donaldson and Wilson, 1975; Holtzer et al., 1955). Due to the striking similarity between

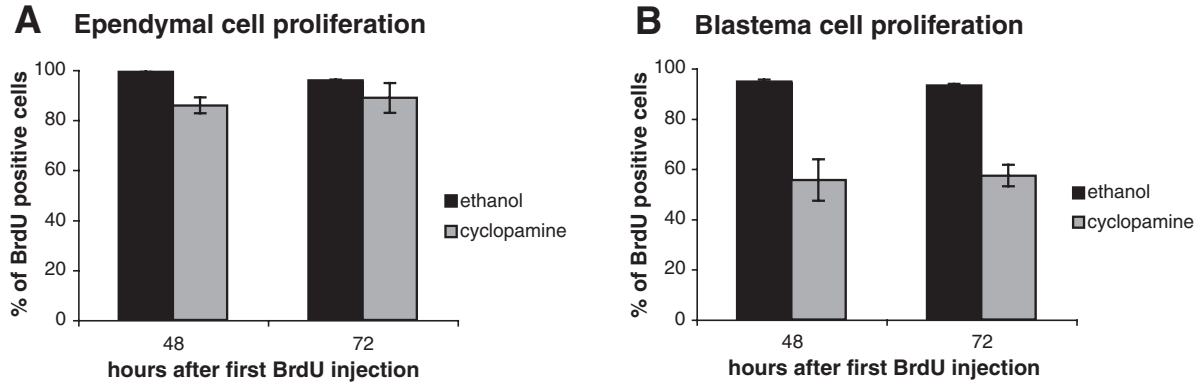

Fig. 6. Hedgehog signaling controls blastema cell proliferation. (A,B) Cumulative BrdU labeling of ependymal cells (A) and ventral blastema cells (B). Each column represents the mean percentage of BrdU-positive cells of 2-4 regenerates at indicated time intervals of BrdU labeling. Between 76 and 255 ependymal and blastema cells were counted in total per regenerate. Error bars are standard deviations. The *t*-test values are 0.005 at 48 hours and 0.212 at 72 hours in A, and 0.003 at 48 hours and 0.002 at 72 hours in B.

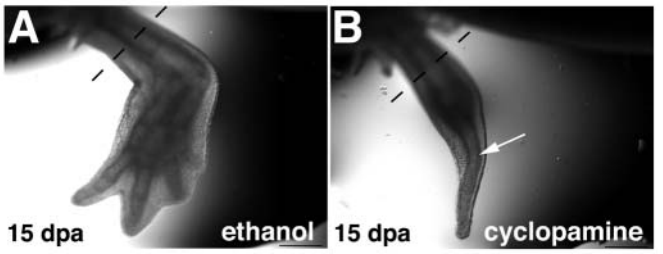

Fig. 7. Cyclopamine treatment of the regenerating axolotl limb does not affect blastema growth but leads to digit loss. (A) The regenerated control limb 15 dpa. (B) The regenerated cyclopamine-treated limb structure 15 dpa. The arrow in B points to the regenerated rod of cartilage. The dashed line in A and B marks the amputation plane. Scale bar: 0.5 mm.

spinal cord removal and the cyclopamine effect on tail regeneration, we wanted to test whether hedgehog signaling is the sole spinal cord signal required for blastema growth and thus tail regeneration. We removed the spinal cord from the tip of the axolotl tail, amputated through the tail devoid of spinal cord, and treated animals with the hedgehog agonist. No regeneration occurred in tails without spinal cord, either in the presence or absence of agonist (Fig. 8A-C). This result indicates that hedgehog signaling is not sufficient to rescue tail regeneration in the absence of the spinal cord. At least one other factor must exist in the spinal cord that is required for tail regeneration.

Discussion

We have examined the DV patterning information that is present in the mature axolotl spinal cord and its transmission to the regenerate. This work has uncovered a role for hedgehog signaling, not only in DV patterning of the spinal cord and surrounding tissues such as cartilage, but also in blastema cell proliferation. We have shown that *Shh*, *Pax6*, Pax7 and *Msx1* were expressed in the differentiated axolotl spinal cord in DV domains similar to those found in development, and that this expression persisted in the ependymal tube throughout tail regeneration. This represents the first time that the patterning information present in the urodele mature tissue and required for regeneration has been localized on a molecular and cellular level. One question that arises is how these domains are 'propagated' along the growing ependymal tube. Several pieces of evidence suggest that signaling within the spinal cord is important for the correct propagation of the domains during regeneration. (1) DV rotation of the mature spinal cord resulted in a rotated orientation of the regenerated spinal cord, indicating that the DV patterning information in the regenerate comes from the mature spinal cord. (2) Lineage tracing experiments indicated that the DV progenitor cell domains in the axolotl spinal cord are not transmitted to the regenerating tail by simple lineage restriction, as 30% of clones from a single ependymal cell spanned multiple DV domains (L. Mchedlishvili, A. Telzerow, H. H. Epperlein and E.M.T., unpublished). (3) The evidence presented in this paper indicates that Shh is expressed in the ventral floor plate of the mature and regenerating spinal cord. Inhibition of hedgehog signaling during tail regeneration caused an expansion of dorsal neural progenitor domains, while activation of hedgehog signaling induced a reduction of dorsal progenitor domains. This indicates that ventral Shh expression in the regenerating spinal cord controls DV progenitor cell identity during tail regeneration. How the *Shh* expression domain itself is maintained in the mature spinal cord and established in the regenerating tube is not yet clear. Taken together, these data strongly indicate that, although during development the initial DV patterning of the neural tube is imposed from structures outside the neural tube, in regeneration the source of ventral Shh signaling comes from within the regenerating spinal cord.

The molecular circuitry controlling DV spinal cord patterning during regeneration

In these experiments, we tested the role of hedgehog signaling by bathing the animals in a uniform concentration of the inhibitor, cyclopamine or the agonist, Hh-Ag1.9. Cyclopamine alone caused ventral expansion of dorsal neural progenitor markers such as Pax7, while agonist alone caused the disappearance of the Pax7 domain, presumably representing a severe ventralization of the ependymal tube. Interestingly, the combination of cyclopamine and agonist restored a relatively normal Pax7 domain, and indeed, normal growth and DV patterning of the entire tail. On the surface it may seem surprising that restoration of normal patterning occurs in response to uniform application of an inhibitor and agonist to a morphogen that clearly acts in a concentration-dependent manner (Briscoe et al., 1999; Ericson et al., 1997a; Ericson et al., 1997b; Roelink et al., 1995). It should be understood, however, that these pharmacological treatments are superimposed on the normal expression of endogenous morphogens such as Shh and presumably Bmp family members. For example, we did not see an alteration of Shh

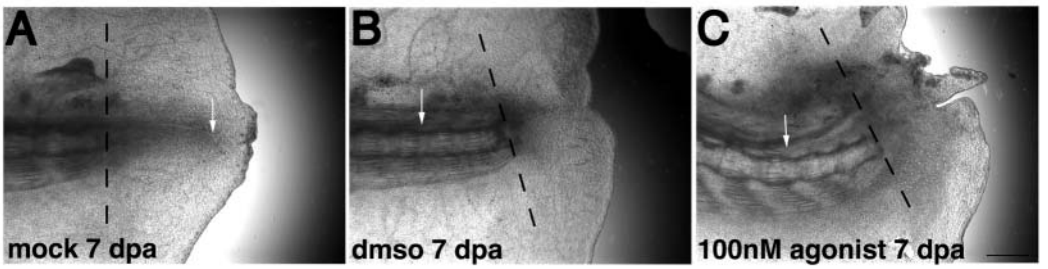

Fig. 8. Hedgehog signaling is not sufficient for tail regeneration in the absence of the spinal cord. (A) Mock operated axolotl tail shows normal 7-day regenerate. (B) Control tail with spinal cord removed does not regenerate. (C) Agonist-treated tail without spinal cord also does not regenerate. Arrows point to the distal tip of the spinal cord in all panels. The dashed line marks the amputation plane. Scale bar: 0.5 mm.

expression in the presence of cyclopamine. This means that endogenous gradients of hedgehog and Bmp are probably still functioning in the face of uniform chemical agents. It is therefore likely that the inhibitor/agonist co-treatment uniformly balances out inhibition and activation of the hedgehog pathway, allowing the endogenous concentration-dependent signaling to be manifested.

In terms of establishing the various DV progenitor cell domains within the spinal cord, a relatively detailed understanding has been gained in embryonic studies, and we assume that the same signaling networks are implemented during regeneration. In particular, Briscoe et al. (Briscoe et al., 2000) have suggested a model to explain how Shh signaling from the floor plate could result in the establishment of distinct neural progenitor domains along the DV axis of the neural tube. Graded Shh signaling results in the definition of two distinct types of molecular domains. The expression of so-called class I homeodomain proteins such as Pax7, Irx3, Dbx1, Dbx2 and Pax6 (found in dorsolateral regions) are repressed by Shh signaling, while expression of class II homeodomain proteins including Nkx6.1 and Nkx2.2 are activated by Shh signals. Cross-repressive interactions between class I and class II homeodomain proteins, such as those between Pax6 and Nkx2.2 (Briscoe et al., 2000), establishes, refines and stabilizes the progenitor cell domains. Although a specific class II protein that represses Pax7 has not been identified yet, presumably additional class II proteins may exist (Briscoe et al., 2000). Therefore, in our case the ventral expansion of Pax7 in cyclopamine-treated regenerates is probably due both to an increase in Pax7 expression stemming from reduced hedgehog signaling, and a decrease in the level of class II proteins that require hedgehog signaling for their expression and that act by restricting Pax7 expression to a dorsal domain. Conversely, reduction of Pax7 in ependymal tubes of hedgehog-agonist-treated regenerates might be due to both an increase in hedgehog signals and in the level of class II proteins that subsequently repress Pax7 in the dorsal tube.

During development, Bmps in the dorsal ectoderm and roof plate are crucial morphogens for DV neural tube patterning. We surmise that Bmp4 and Wnt3a are expressed in the dorsal axolotl spinal cord. Although we could detect Bmp4 and Wnt3a in tail blastema RNA by RT-PCR, attempts to localize Bmp4 and Wnt3a by in situ hybridization or phospho-Smad1 immunohistochemistry have so far been unsuccessful. The presence of *Msx1*, a known downstream target of Bmp4 (Liem et al., 1995; Timmer et al., 2002), in the axolotl dorsal spinal cord suggests the presence of Bmp signaling within the spinal cord.

The role of hedgehog signaling in patterning the tail blastema

In addition to the role of hedgehog signaling in patterning the regenerating spinal cord, we have demonstrated that hedgehog is also required for patterning the surrounding blastema tissue. The early cartilage marker Sox9 was not expressed in cyclopamine-treated animals. We favor the idea that this reflects a requirement of hedgehog to induce Sox9 expression rather than complete absence of Sox9-expressing cells in the blastema, for several reasons. First, during development, Shh signaling from the notochord and neural tube induces Pax1, Pax9 and Sox9 in the sclerotome, the precursors for cartilage (Fan and Tessier-Lavigne, 1994; Marcelle et al., 1999; Murtaugh et al., 1999; Tavella et al., 2004; Zeng et al., 2002). In the blastema, the location of Sox9 expression with respect to the regenerating spinal cord is distinct from that during development, as it appears ventral to the ependymal tube rather than in lateral sclerotomal cells. Although regeneration does not proceed through a morphologically distinct somite, it is very likely, however, that the molecular signaling pathway leading to cartilage formation in the two contexts are the same. Second, the hedgehog agonist could induce ectopic Sox9 expression in dorsal regions of the blastema. The fact that only isolated dorsal blastema cells expressed Sox9, rather than massive formation of cartilage throughout the blastema in agonist-treated regenerates, is probably due to the inhibitory role of molecules such as Bmps in the dorsal regenerate that would antagonize the agonist effect.

The role of hedgehog signaling in blastema cell proliferation

A striking aspect of our results is the profound dependence of tail regeneration on hedgehog signaling. BrdU labeling indicated at least a 40% reduction in cycling blastema cells. This result probably represents an underestimate, because it is difficult to distinguish the cycling fin cells from the blastema cells due to lack of a blastema cell marker. We favor the idea that sonic hedgehog is a direct mitogen for blastema cells, as the patched receptor is expressed in the blastema, although it is possible that hedgehog expression in the regenerate may be necessary for the expression of a blastema cell mitogen. For example, in the limb sonic hedgehog expression upregulates Fgf4 in the apical ectodermal ridge to promote limb bud outgrowth (Laufer et al., 1994; Niswander et al., 1994). We have tested the role of signaling through the Fgfr1 in tail regeneration and found that it cannot account for the effect of hedgehog. While chemical inhibition of Fgfr1 signaling during tail regeneration via the pharmacological inhibitor SU5402 initially slowed down regeneration slightly, the regenerated tails showed no other phenotype and grew to a normal length (data not shown) – a phenotype quite distinct from hedgehog inhibition. It appears, however, that hedgehog is not the sole factor required for blastema cell proliferation, as the hedgehog agonist could not rescue the blastema growth defect produced by spinal cord removal.

Concluding remarks

Our study also has implications for understanding the origin and fate of blastema cells. As Shh is already expressed in the mature spinal cord, the tail blastema cells receive signals that direct them to specific cell fates as soon as they are born. A naive blastema cell may therefore be extremely difficult to detect. Although the ventral blastema cells behave similarly to sclerotome, it is not clear if an early blastema cell that responds to the Shh signal is equivalent to an early somite cell, a presomitic mesoderm cell, or is a completely distinctive cell type. It is possible, for example, that the blastema cell has more fates available to it than a typical sclerotomal cell. Furthermore, it is unclear if the Sox9-expressing blastema cells derive solely from sclerotomal derivatives in the mature tissue, or whether different tissue types can contribute blastema cells that are induced to express Sox9. Echeverri and Tanaka (Echeverri and Tanaka, 2002) showed that cells can migrate

from the spinal cord and contribute to cartilage during tail regeneration, indicating that cartilage precursors have diverse origins. Specific labeling of different cell types in the mature tissue and long-term lineage tracing will be required to fully address this issue.

It is noteworthy that the role of hedgehog signaling during axolotl limb regeneration is clearly different from its role in tail regeneration. In the limb blastema, Shh controlled AP digit formation and did not severely inhibit blastema outgrowth or cartilage formation. This indicates that blastema cells probably have region-specific identities that allow them to respond to inductive cues in distinct ways. Presumably this region-specific identity is maintained in the mature tissue and inherited by blastema cells, although it is possible that such identity is positively reinforced during regeneration, and that in certain cases, this identity could be reversed.

The maintenance of patterning information in the mature tissue may be a central feature of regenerative ability. Adult mouse and chick spinal cord tissue does not maintain the markers examined here, and this may represent a block for regeneration (Fu et al., 2003; Yamamoto et al., 2001). Interestingly, injury of the mouse spinal cord did result in the appearance of Pax7-positive cells in the parenchyma of the dorsal horn (Yamamoto et al., 2001). These Pax7-positive cells co-stained with nestin, indicating that they may have the capacity to act as progenitor cells. Such observations suggest the possibility that mammals harbor a latent capacity to re-induce important aspects of cell patterning after injury. The comparison of patterning marker expression in spinal cord progenitor cells (and other tissues) in different species may be an important dimension of understanding the regenerative ability.

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