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## Sonic hedgehog is a polarized signal for motor neuron regeneration in adult zebrafish

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### SUMMARY

In contrast to mammals, the spinal cord of adult zebrafish has the capacity to reinitiate generation of motor neurons after a lesion. Here we show that genes involved in motor neuron development, i.e. the ventral morphogen *sonic hedgehog a (shha)*, as well as the transcription factors *nkx6.1* and *pax6*, together with a *Tg(olig2:egfp)* transgene, are expressed in the unlesioned spinal cord of adult zebrafish. Expression is found in ependymo-radial glial cells lining the central canal in ventro-dorsal positions that match expression domains of these genes in the developing neural tube. Specifically, *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells, the adult motor neuron progenitors, co-express *Nkx6.1* and *Pax6*, thus defining an adult motor neuron progenitor (pMN)-like zone. *Shha* is expressed in distinct ventral ependymo-radial glial cells. After a lesion, expression of all these genes is strongly increased, while relative spatial expression domains are maintained. In addition, expression of the hedgehog (hh) receptors *patched1* and *smoothed* becomes detectable in ependymo-radial glial cells including those of the pMN-like zone. Cyclopamine-induced knock down of hh signaling significantly reduces ventricular proliferation and motor neuron regeneration. Expression of indicator genes for the FGF and retinoic acid signaling pathways was also increased in the lesioned spinal cord. This suggests that a sub-class of ependymo-radial glial cells retain their identity as motor neuron progenitors into adulthood and are capable of reacting to an shh signal and potentially other developmental signals with motor neuron regeneration after a spinal lesion.

### Keywords

stem cells; radial glia; cyclopamine; BrdU; PCNA; *nkx6.1*; *pax6*; FGF; retinoic acid

### INTRODUCTION

Adult zebrafish, in contrast to mammals (Ninkovic and Götz, 2007; Bareyre, 2008), regenerate severed axons from the brainstem (Becker et al., 1997) and regain motor function (van Raamsdonk et al., 1998; Becker et al., 2004) after a spinal transection lesion. Spinal motor neurons that are lost after a lesion also regenerate (Reimer et al., 2008), similar to other teleost species (Kirsche, 1950). Initially, newly generated motor neurons are clearly discernable from resident motor neurons by their small diameter. However, regenerated motor neurons in zebrafish fully mature as indicated by expression of choline acetyl

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transferase at later stages. Moreover, elaboration of peripheral axons and soma coverage with synaptic markers suggest network integration of regenerated motor neurons. Lineage tracing experiments indicate that regenerated motor neurons likely derive from proliferating *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells, which line the central canal and contact the pial surface with their endfeet (Reimer et al., 2008). Under non-lesion conditions these cells only produce oligodendrocytes (Park et al., 2007). In the spinal cord of adult mammals, similar cells with stem cell potential exist (Shihabuddin et al., 2000; Meletis et al., 2008). However, motor neuron differentiation has never been observed in vivo (Ohori et al., 2006; Meletis et al., 2008).

To elucidate how motor neuron regeneration in adult zebrafish is induced, we decided to investigate the role of the transcription factors *nkx6.1*, *pax6* and *olig2*, as well as hedgehog (hh) signaling. These factors are crucial for motor neuron generation in the developing spinal cord (Ingham and McMahon, 2001; Varjosalo and Taipale, 2008), together with FGF and retinoic acid signaling (Novitsch et al., 2003). Moreover, Shh is important for adult neurogenesis in the mammalian forebrain (Lai et al., 2003; Machold et al., 2003).

Hh family genes are expressed in the embryonic floor plate (*shha* and *shhb* in zebrafish) and instruct the formation of transcription factor domains along the ventro-dorsal axis in the spinal cord (Krauss et al., 1993; Currie and Ingham, 1996; Avaron et al., 2006). The ventro-lateral motor neuron progenitor (pMN) domain expresses a combination of *nkx6.1*, *pax6* and *olig2* in all vertebrates, including zebrafish, and gives rise to motor neurons that express to transcription factors *hb9* and *islet-1/-2* (Jessell, 2000; Cheesman et al., 2004; Park et al., 2004; Fuccillo et al., 2006).

Hhs act by binding to the receptor Patched1, leading to de-repression of the transmembrane protein Smoothened, which in turn leads to Gli mediated activation of target genes. These include *patched1* itself as part of an autoregulatory feedback loop in zebrafish (Concordet et al., 1996) and other vertebrates (Dessaud et al., 2008).

We find *shha* expressing and pMN-like ependymo-radial glial cells (defined by expression of *Tg(olig2:egfp)*, Nkx6.1 and Pax6) in the unlesioned adult spinal cord, despite the absence of adult motor neuron generation. However, during lesion induced motor neuron regeneration, numbers of pMN-like ependymo-radial glial cells and expression levels of *shha*, *patched1*, *smoothened*, *olig2*, Nkk6.1 and Pax6, as well as FGF and retinoic acid pathway genes are greatly enhanced. Blocking hh signaling in adult fish in vivo impairs motor neuron regeneration. This suggests an important role for Shh and possibly other developmental signals in adult motor neuron regeneration.

## EXPERIMENTAL PROCEDURES

### Animals

All fish are kept and bred in our laboratory fish facility according to standard methods (Westerfield, 1989) and all experiments have been approved by the British Home Office. We used wild type (wik), *Tg(hb9:gfp)* (Flanagan-Steet et al., 2005), *Tg(olig2:egfp)* (Shin et al., 2003) and *Tg(shha:gfp)* (Shkumatava et al., 2004) transgenic fish.

### Spinal cord lesion

As described previously (Becker et al., 1997), fish were anesthetized by immersion in 0.033% aminobenzoic acid ethylmethylester (MS222; Sigma, St. Louis, MO) in PBS for 5 min. A longitudinal incision was made at the side of the fish to expose the vertebral column. The spinal cord was completely transected under visual control 4 mm caudal to the brainstem-spinal cord junction.

### Intraperitoneal substance application

Animals were anaesthetized and intraperitoneally injected. Cyclopamine was purchased from LC Laboratories (Woburn, MA, USA). Specific activity of cyclopamine was tested by incubating embryos with the substance, as describe elsewhere (Park et al., 2004). This treatment resulted in cyclopia and loss of motor axons. The related control substance tomatidine (Sigma-Aldrich, UK) had no effect (data not shown). For intraperitoneal injections into adult fish, cyclopamine and tomatidine were dissolved in 45% (2-Hydroxypropyl)-beta-cyclodextrin (Sigma-Aldrich, UK) and injected at a concentration of 0.2 mg/ml in a volume of 25  $\mu$ l (equaling 10 mg/kg, Sanchez and Ruiz i Altaba, 2005) at 3, 6 and 9 days post-lesion. Analysis took place at 14 days post-lesion.

### Immunohistochemistry

We used mouse-anti Pax6 (kindly provided by V. van Heyningen) and rabbit anti-Pax6 (Covance, 1:300; both Pax6 antibodies showed identical results), mouse anti-Nkx6.1 (AB2024, 1:1000; kindly provided by O. Madsen, Hagedorn Research Institute, Gentofte, Denmark and purchased from the Developmental Studies Hybridoma Bank, University of Iowa, F55A10) and mouse anti-PCNA (PC10, 1:500, Dako Cytomation, Glostrup, Denmark) antibodies. Secondary Cy2-, Cy3- and Cy5-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Animals were transcardially perfused with 4% paraformaldehyde and post-fixed at 4°C overnight. Spinal cords were dissected and floating sections (50  $\mu$ m thickness) were produced with a vibrating blade microtome (Microm, Volketswil, Switzerland). Antigen retrieval was carried out by incubating the sections for 1 hour in citrate buffer (10mM sodium citrate in PBS, pH=6.0) at 85°C for 30 minutes for Nkx6.1, Pax6 and PCNA immunohistochemistry. All other steps were carried out in PBS (pH 7.4) containing 0.1% triton-X100. Sections were blocked in goat serum (15  $\mu$ l/ml) for 30 minutes, incubated with the primary antibody at 4°C overnight, washed three times 15 minutes, incubated with the appropriate secondary antibody for 1h, washed again, mounted in 70% glycerol and analyzed using a confocal microscope (Zeiss Axioskop LSM 510). Co-labeling of cells was always determined in individual optical sections.

### In situ hybridisation

We used previously published probes to detect *shha* (Krauss et al., 1993), *patched1* (Concordet et al., 1996), *smoothed* (Varga et al., 2001) and *olig2* (Park et al., 2002) mRNAs. The in situ hybridization procedure on vibratome sections (50  $\mu$ m thickness) followed a previously published protocol (Lieberoth et al., 2003).

### Retrograde axonal tracing

Retrograde axonal tracing from a spinal level 3.5 mm caudal to the transection site has been described (Becker et al., 1997). Briefly, biocytin was applied to the spinal cord and the tracer was allowed to be retrogradely transported to the somata of brainstem neurons with regenerated spinal axons overnight. Fish were perfused and labeled profiles were analyzed in vibratome sections (50  $\mu$ m thickness) of the entire brain.

### Behavioral assays of functional recovery

We tested the endurance of unlesioned and lesioned fish by recording the time they were able to keep their position while swimming against a water current in an adaption of a published protocol (van Raamsdonk et al., 1998). In a tunnel with a flat bottom (7 cm width), 15 cm long compartments were divided off by wire mesh. A current of 15 cm/sec was induced using a pond pump (Nautilus 8000, Oase, Hörstel, Germany). The time fish were able to withstand the water current was recorded for up to 3 hours, when full recovery

was assumed. Unlesioned fish always endured for 3 hours. Lesioned fish showed gradual recovery between 1 and 6 weeks post-lesion, in agreement with previous tests of recovery of unforced swimming behavior (Becker et al., 2004).

### Reverse transcriptase (RT-) PCR

To assess the effect of cyclopamine on hh down-stream genes spinal tissue was collected from the area of 3 mm surrounding the lesion site and RNA was extracted (RNeasy Mini Kit, Qiagen, UK) from pooled tissue of at least 5 animals per treatment at 5 days post-lesion from animals that had received a single cyclopamine or tomatidine injection at 4 days post-lesion. Reverse transcription, using random primers (Promega, Madison, WI, USA), was performed with the SuperScript III kit (Invitrogen, UK). The following primers were used to amplify *patched1* and *olig2*, normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by standard PCR (57°C, 27 cycles for *olig2* and 30 cycles for *patched1*): GAPDH forward: 5'-ACTCCACTCATGGCCGTTAC-3', GAPDH reverse: 5'-TCTTCTGTGTGGCGGTGTAG-3', *Olig2* forward: 5'-TCCAGCAGACCTTCTTCTCC-3', *Olig2* reverse: 5'-ACAAGTGGACGGATGGAAACC-3', *patched1* forward: 5'-GTC TGC AAG CCA CTT TTG ATG C -3', *patched1* reverse: 5'-GGG GTA GCC ATT GGG ATA GT -3'.

To assess expression levels of FGF and retinoic acid pathway related genes, cDNA was prepared from unlesioned and lesioned (14 days post-lesion) spinal cords in the same way as above. Standard PCR (35 cycles) was performed using primers and temperatures indicated in suppl. Table 1.

### Cell counts and statistical analysis

Numbers of small motor neurons around the lesion site in *Tg(hb9:gfp)* fish were determined from stereological counts in confocal image stacks of three randomly selected vibratome sections from the region up to 750 µm rostral to the lesion site and three sections from the region up to 750 µm caudal to the lesion site. Cell numbers were then calculated for the entire 1.5 mm surrounding the lesion site. PCNA<sup>+</sup> cell profiles were counted in at least 6 randomly selected sections per animal from the same area by fluorescence microscopy. The observer was blinded to experimental treatments.

To estimate the increase in the number of ventricular cells expressing transcription factors after a lesion we counted labeled nuclear profiles in an optical section (0.4 µm in thickness), which showed clear labeling, within 750 µm rostral to caudal of the lesion site. Parenchymal cells were scored in a square area of 146 µm edge length, centered around the central canal.

Variability of values is given as standard error of the mean. Statistical significance was determined using the Mann-Whitney U-test ( $P < 0.05$ ) unless indicated differently.

## RESULTS

We aimed to elucidate the set of transcription factors that are expressed during motor neuron regeneration in the spinal cord of adult zebrafish and the signals that are active during regeneration using known developmental mechanisms as a starting point.

### Expression of pMN transcription factors is retained in adult zebrafish and is increased after a spinal lesion

In the developing neural tube, motor<sup>+</sup> neurons are derived from an *olig2*<sup>+</sup>, *nkx6.1*<sup>+</sup> and *pax6*<sup>+</sup> domain (Fig. 1A). Therefore, we analyzed expression of these genes in the adult spinal cord in the unlesioned and lesioned situation, focusing on the ventricular zone of *Tg(olig2:egfp)*<sup>+</sup>

ependymo-radial glial cells from which motor neurons regenerate (Reimer et al., 2008). We found that, generally, the ventricle increased massively in size close to the lesion site. The average circumference of the central canal in cross section within 750  $\mu\text{m}$  of the lesion site increased to almost 300% at 2 weeks post-lesion (unlesioned:  $33.4 \pm 1.60$ ,  $n = 7$  animals; lesioned:  $97.7 \pm 10.50$   $\mu\text{m}$ ,  $n = 7$  animals; ANOVA,  $P = 0.0001$ ; Fig. 1B) and remained enlarged at 6 weeks post-lesion ( $89.5 \pm 7.91$   $\mu\text{m}$ ,  $n = 7$  animals; ANOVA,  $P = 0.0002$ ). It has been found in the eel that widening of the central canal after spinal cord lesion directly correlates with an increase in the number of ventricular cells (Dervan and Roberts, 2003). Ventricular cells are potential stem cells and are the major proliferating cell type in the lesioned spinal cord (Reimer et al., 2008). Thus, a widened ventricle suggests an increase in symmetrical cell division of these cells that may lead to larger domains of progenitor cells.

Using in situ hybridization for *olig2* mRNA, we found that expression was undetectable in the unlesioned spinal cord. After a lesion, expression of *olig2* mRNA was not detectable at 1 week post-lesion. At 2 weeks post-lesion, mRNA expression was strongly increased in the ependymal zone in a ventro-lateral position, comparable to the relative position of developmental expression in the neural tube (Fig. 1A), in the vicinity of the lesion site (250  $\mu\text{m}$  rostral and caudal to it; Fig. 1B). Expression of *olig2* mRNA was back to undetectable control levels at 6 weeks post-lesion. Thus spatial and temporal regulation of *olig2* coincided with the timing and location of motor neuron regeneration after a lesion, which also peaks close to the lesion site at 2 weeks post-lesion (Reimer et al., 2008).

To determine the cell type that expresses *olig2* mRNA, we used a *Tg(olig2:egfp)* transgenic fish. The transgene labels ependymo-radial glial cells and oligodendrocytes in the unlesioned and lesioned spinal cord (Park et al., 2007; Reimer et al., 2008). The ventricular position of transgenic ependymo-radial glial cells matched that of *olig2* mRNA expressing cells. The domain of *Tg(olig2:egfp)*<sup>+</sup> cells at the ventricle was expanded in the vicinity of the lesion site by almost 80% (unlesioned:  $10.3 \pm 0.50$   $\mu\text{m}$ ,  $n = 6$  animals; lesioned:  $18.0 \pm 3.07$   $\mu\text{m}$ ,  $n = 7$  animals,  $P = 0.0183$ ; Fig. 2) at 2 weeks post-lesion.

While transgene expression closely matched that of the mRNA in the lesioned situation, only the transgene was detectable in ventro-lateral ependymo-radial glial cells in the unlesioned situation. We cannot exclude that the *Tg(olig2:egfp)* transgene expression in pMN-like cells in the unlesioned spinal cord is non-specific. However, *Tg(olig2:egfp)* expression is precisely localized in the zone of Nkx6.1+/Pax6+ ependymo-radial glial cells in the ventro-lateral spinal cord (see below) and the transgene is driven by a large, reliable promoter region contained in a BAC clone (Shin et al., 2003). Thus it is possible that, by a cumulative effect of the highly stable GFP (Cubitt et al., 1995), the transgene may report levels of gene expression that were below the detection level of in situ hybridization. Thus a *Tg(olig2:egfp)* transgene is expressed in ventro-lateral ependymo-radial glial cells of the unlesioned spinal cord, and *olig2* mRNA expression is clearly increased in that position in response to a lesion.

Nkx6.1 immunoreactivity was found in a U-shaped pattern in ventricular cells around the ventral central canal in unlesioned adult animals, which is reminiscent of the embryonic expression pattern in the neural tube. Some parenchymal cells were also Nkx6.1<sup>+</sup>. At 2 weeks after a spinal lesion, Nkx6.1 immunoreactivity was still detectable in a U-shape around the ventricle. However, more Nkx6.1<sup>+</sup> ventricular cells were present and immunoreactivity was more intense. The number of ventricular Nkx6.1 immunoreactive nuclear profiles in single optical sections increased from  $7.5 \pm 1.45$  profiles/section ( $n = 6$  animals) to  $35.3 \pm 5.58$  at 2 weeks post-lesion ( $n = 6$  animals,  $P = 0.0039$ ). In the parenchyma, we observed  $4.2 \pm 0.83$  cells in unlesioned animals and  $6.3 \pm 1.02$  profiles/section in lesioned animals;  $P = 0.16$ ). In multiple labeling experiments, we found that



Nkx6.1 immuno-reactivity overlapped with the *Tg(olig2:egfp)*<sup>+</sup> zone of the adult ventricle and exceeded it slightly dorsally in the unlesioned and lesioned spinal cord (Fig. 2). Thus Nkx6.1 is expressed at low levels in the ventral ventricular zone of the unlesioned adult spinal cord and its expression is increased in the same relative position after a lesion.

Pax6 immunoreactivity was detectable in an inverted U-shape around the ventricle and in some parenchymal cells in unlesioned animals. There was a ventral-high to dorsal-low gradient in Pax6 immunoreactivity. At 2 weeks post-lesion this expression pattern was retained (Fig. 2). However, the number of ventricular Pax6 immunoreactive profiles in single optical sections strongly increased from  $9.3 \pm 2.19$  profiles/section ( $n = 6$  animals) to  $43.5 \pm 13.13$  at 2 weeks post-lesion ( $n = 6$  animals,  $P = 0.0039$ ). In the parenchyma, we observed  $3.7 \pm 0.80$  profiles/section in unlesioned animals and  $8.3 \pm 1.15$  profiles/section in lesioned animals ( $P = 0.0096$ ), indicating a moderate increase in the number of parenchymal Pax6<sup>+</sup> cells. Co-labeling indicated that Pax6 immunoreactivity at the central canal overlapped with the *Tg(olig2:egfp)*<sup>+</sup> zone and shared a ventral border with it in the unlesioned and lesioned spinal cord. Ventral Pax6 immunoreactivity also overlapped with dorsal Nkx6.1 immunoreactivity (Fig. 2). Thus, Pax6 expression in the latero-dorsal aspect of the spinal cord is increased after a spinal lesion.

Overall, triple-labeling indicated that the motor neuron-generating *Tg(olig2:egfp)*<sup>+</sup> zone was also Pax6<sup>+</sup> and Nkx6.1<sup>+</sup>. Individual *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells were found to co-express both Nkx6.1 and Pax6 in single optical sections of the unlesioned and lesioned spinal cord and their number was increased from  $2.0 \pm 0.00$  profiles/section in unlesioned animals ( $n = 2$ ) to  $13.7 \pm 2.19$  profiles/section at 2 weeks post-lesion ( $n = 3$  animals). Parenchymal cells were rarely found to co-express *Tg(olig2:egfp)*, Nkx6.1 and Pax6 (unlesioned: 0 profiles/section; lesioned:  $0.3 \pm 0.33$  profiles/section). Thus a subset of ependymo-radial glial cells in the adult spinal cord retain low, but clearly detectable, expression of markers for the pMN zone, namely *Tg(olig2:egfp)*, Nkx6.1 and Pax6 in specific domains. The number of cells co-expressing these genes and gene expression levels are increased, when motor neurons are generated after a lesion (Reimer et al., 2008).

### ***Shha* is upregulated in a distinct class of proliferating ependymo-radial glial cells at the ventral midline of the spinal cord**

To determine the possible signals that induce adult pMN-like ependymo-radial glial cells to generate motor neurons we analyzed expression of *shha*. In the unlesioned adult spinal cord, there were very few cells contacting the central canal at the ventral midline that expressed *shha* mRNA, as detected by in situ hybridization. At 1 week post-lesion, this pattern was unchanged. At 2 weeks post-lesion, the area and labeling intensity of the signal at the ventral midline was strongly increased in a gradient with strongest expression in the immediate vicinity of the lesion site (Fig. 3A). At a distance of more than 1 mm rostral or caudal to the lesion site, labeling intensity was not different from unlesioned controls. At 6 weeks post-lesion, labeling intensity was reduced again, but still higher than in unlesioned controls. This spatio-temporal expression pattern is similar to that of *olig2* mRNA (see above) and the spatio-temporal distribution of motor neuron regeneration (Reimer et al., 2008)

To determine which cell type expressed *shha* we analyzed transgenic fish, in which expression of GFP is under the control of the *shha* promoter. In unlesioned animals, a few ependymo-radial glial cells at the ventral midline of the spinal cord expressed GFP. These cells had cell bodies contacting the ventricle and their radial processes could be followed to the pial surface where their endfeet formed an uninterrupted ventral part of the glial limitans. At 2 weeks post-lesion, these cells increased in number and still formed the glial limitans (Fig. 3A). *Tg(shha:gfp)*<sup>+</sup> ependymo-radial glial cells were proliferating at 2 weeks post-

lesion as indicated by double-labeling with an antibody to PCNA, which labels cells in early G1 phase and S phase of the cell cycle (Fig. 3B).

*Tg(shha:gfp)*<sup>+</sup> ependymo-radial glial cells were distinct from *Tg(olig2:egfp)*<sup>+</sup> motor neuron progenitor cells, as revealed in multiple labeling experiments. Pax6 immunoreactivity bordered ventrally on *Tg(shha:gfp)*<sup>+</sup> ependymo-radial glial cells in the unlesioned and lesioned spinal cord (Fig. 4). *Tg(shha:gfp)*<sup>+</sup>/Pax6<sup>+</sup> cells were not detected, in contrast to *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells, which expressed Pax6 (see Fig. 2). The fact that the ventral border of Pax6 expression in ventricular cells is co-extensive with that of *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells indicates that pMN-like cells line the central canal immediately adjacent to the *shha* expressing cells. In summary, in situ hybridization and analysis of transgene expression indicated that in the lesioned adult spinal cord *shha* expression was increased by proliferating ventral ependymo-radial glial cells, directly adjacent to motor neuron progenitor cells.

### Expression patterns of hh receptors suggests lesion-induced activity of the pathway in motor neuron progenitor cells

If hh signaling is active in the adult lesioned spinal cord, the *patched1* receptor and target gene as well as *smoothened* as co-receptor should be expressed. In situ hybridization indicated that *smoothened* mRNA was undetectable in the unlesioned spinal cord. We found increased expression of *smoothened* mRNA along the entire dorso-ventral extent of the ventricular zone of the lesioned spinal cord in a proximo-distal gradient that was strongest close to the lesion site at 2 weeks post-lesion (Fig. 5A). At a distance of more than 1 mm rostral and caudal to the lesion site no enhanced *smoothened* expression was detectable. This matches the proximo-distal expression gradient of *shha*.

*Patched1* mRNA expression was likewise increased from undetectable levels close to the lesion site at 2 weeks post-lesion. However, the pattern of expression differed from that of *smoothened*. The ventral zone, corresponding to the cells that express *shha*, was free of signal. In the adjacent zone there was strong expression of *patched1* mRNA that tapered off towards the dorsal spinal cord (Fig. 5A). Since strong expression of *patched1* is an indicator of activity of the hh pathway, we determined whether *Tg(olig2:egfp)*<sup>+</sup> motor neuron progenitors expressed *patched1*, using double labeling of *patched1* mRNA in lesioned *Tg(olig2:egfp)* transgenic fish at 2 weeks post-lesion. The region of strongest *patched1* mRNA expression overlapped with *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells, consistent with the hypothesis that Shh-dependent transcription took place in *Tg(olig2:egfp)*<sup>+</sup> motor neuron progenitor cells after a lesion (Fig. 5B).

### Blocking Hh signaling attenuates motor neuron regeneration and proliferation of ventricular cells

To determine whether Shh might influence motor neuron regeneration we blocked hh pathway activity by intraperitoneal injections of the specific inhibitor cyclopamine. Injection of cyclopamine led to a specific reduction in expression of *patched1* and *olig2* mRNA in the lesioned spinal cord relative to the constitutively expressed gene GAPDH at 24 h after the injection, as detected by PCR analysis of the lesioned spinal cord. This indicates that intraperitoneal injections of cyclopamine can indeed reduce expression of Shh target genes in the lesioned spinal cord (Fig. 6).

To determine whether cyclopamine treatment would influence motor neuron regeneration, we used the *Tg(hb9:gfp)* transgenic fish, because newly generated motor neurons can be readily discriminated by transgene expression and their small size (<12 μm diameter) in lesioned animals (Reimer et al., 2008), thus obviating the need for BrdU labeling in this

analysis. The accuracy of transgenic labeling of newly generated motor neurons has previously been confirmed by Hb9 and Islet-1/-2 immunohistochemistry (Reimer et al., 2008). Analysis took place at 2 weeks post-lesion, when motor neuron regeneration peaks. Repeated cyclopamine injections (see methods) caused a highly significant 50% reduction in the number of newly generated *Tg(hb9:gfp)*<sup>+</sup> motor neurons within 1.5 mm surrounding the lesion site ( $377 \pm 45.7$  cells/animal;  $n = 9$  animals) compared with animals injected with the related, but ineffective substance tomatidine ( $747 \pm 42.2$  cells/animal;  $n = 10$  animals;  $P = 0.0004$ ; Fig. 7A,B) in stereological counts. In unlesioned animals, the number of small *Tg(hb9:gfp)*<sup>+</sup> motor neurons is very low ( $20 \pm 7.7$  cells/animal, Fig. 7 and Reimer et al., 2008). This suggests that hh signaling is necessary for efficient motor neuron regeneration.

Since Shh is a mitogen (Jessell, 2000; Fuccillo et al., 2006), we tested whether cyclopamine would attenuate proliferation in the lesioned spinal cord. In the unlesioned spinal cord, proliferation is very low (Fig. 7). Lesion-induced proliferation occurs mostly at the ventricle, peaks at 2 weeks post-lesion and can be detected using PCNA antibodies (Reimer et al., 2008). Indeed, the number of PCNA<sup>+</sup> cell profiles in the ventricular zone of cyclopamine-injected animals ( $45 \pm 2.8$  profiles/section;  $n = 16$  animals) was significantly reduced by 25% compared with tomatidine-injected control animals ( $60 \pm 7.0$  profiles/section;  $n = 10$  animals;  $P = 0.027$ ; one-tailed test; Fig. 7A,B) at 2 weeks post-lesion. This indicates that proliferation of progenitor cells at the ventricle depends at least in part on hh signaling.

### **Attenuation of motor neuron regeneration does not impair functional recovery or axon regrowth from the brainstem**

The possibility to reduce motor neuron regeneration around a spinal lesion with cyclopamine afforded the opportunity to test whether these neurons contribute to recovery of swimming behavior. We performed a test of swimming performance at 6 weeks post-lesion, when functional recovery plateaus (Becker et al., 2004). We adapted an endurance test in which fish swim against a constant water current (van Raamsdonk et al., 1998). Cyclopamine-injected animals reached a mean endurance of  $77 \text{ min} \pm 36.3 \text{ min}$  ( $n = 15$  animals), whereas tomatidine-injected animals reached  $80 \pm 36.6 \text{ min}$  ( $n = 20$  animals) at six weeks post-lesion (from less than 15 seconds for both groups at 7 days post-lesion). While both experimental groups showed significant improvement of endurance over time (ANOVA,  $p < 0.0001$ ), the slightly lower endurance in cyclopamine treated fish was not statistically significant compared to control treatment ( $P = 0.72$ ). Another test in which the distance swum by freely moving fish within a given time frame (Becker et al., 2004) did also not yield significant differences between tomatidine and cyclopamine treated animals (data not shown).

We tested whether cyclopamine injections would influence axon regrowth from the brainstem, which is essential for functional recovery (Becker et al., 2004). The number of supraspinal neurons that grew an axon beyond the spinal lesion site at 6 weeks post-lesion was also not significantly impaired by cyclopamine injections (cyclopamine:  $58 \pm 10.0$  neurons,  $n = 3$ ; control:  $66.6 \pm 8.5$  neurons,  $n = 9$ ,  $P = 0.52$ ). Thus a 50% reduction of motor neuron regeneration around a spinal lesion site by cyclopamine did not detectably impair functional recovery or regrowth of axons from the brainstem.

### **Genes indicating FGF and retinoic acid pathway activity show increased expression in the lesioned spinal cord**

Cyclopamine treatment did not completely inhibit motor neuron regeneration. This suggests involvement of other signaling pathways in adult motor neuron regeneration. To elucidate such signals we analyzed the expression of indicator genes for the retinoic acid and FGF pathways in the adult regenerating spinal cord, because these signaling pathways are known



to play a role during embryonic motor neuron generation (Novitch et al., 2003). For the FGF pathway we found that mRNA expression of *fgf3* and the down-stream gene *sprouty4* (*spry4*) was robustly increased in the lesioned spinal cord (Fig. 8). Other signals (*fgf8*, *fgf17b*), receptors (*fgfr1a*, *fgfr2*, *fgfr3*, *fgfr4*) and down-stream genes (*dusp6*, *pea3*, *spry1*, *spry2*) showed little or no upregulation (suppl. Fig 1). For the retinoic acid pathway we detected a robust increase in mRNA levels of retinoic acid receptor subunits (*rarab*, *rxrga*, *rxrgb*) and down-stream genes (*crabp2a*, *cyp26a*) (Fig. 8). The retinoic acid synthesizing enzyme *raldh2*, as well as other receptor subunits (*raraa*, *rarga*, *rargb*, *rxraa*) and the down-stream gene *crabp2b* showed little or no increase in mRNA expression (suppl. Fig. 1). These findings suggest that FGF and retinoic acid signaling could also be involved in motor neuron regeneration.

## DISCUSSION

In this study we demonstrate for the first time that the myelinated, fully adult spinal cord of zebrafish contains a subpopulation of ependymo-radial glial cells that retain transcription factor expression of the embryonic pMN zone. We provide evidence that these cells, similar to their embryonic counterparts, react to an Shh signal from the ventral spinal cord with motor neuron production after a spinal lesion (summarized in Fig. 9).

### Evidence for a role of Shh in motor neuron regeneration

Upregulation of *patched1* in pMN-like ependymo-radial glial cells may indicate lesion-induced activity of Shh signaling in these cells. This is because *patched1* is not only a receptor, but also a target gene of the hh pathway, thus reporting activity of the pathway (Concordet et al., 1996; Machold et al., 2003). Indeed, *patched1* shows a clear gradient from ventral-strong to dorsal-weak expression, with strongest expression in pMN-like ependymo-radial glial cells, which are closest to the source of *shha* expression. In contrast, *smoothened*, which is also necessary for hh signaling, but not a direct target of the pathway, is uniformly expressed in ependymo-radial glial cells along the dorso-ventral axis of the lesioned spinal cord.

Inhibition of hh signaling with cyclopamine indicates that the pathway plays a major role in motor neuron regeneration. Blocking the signal with cyclopamine in vivo reduced motor neuron regeneration by 50%. The fact that ventricular cell division was also reduced suggests that Shh may act at the level of cell division in pMN-like ependymo-radial glial cells. However, effects on the acquisition of a motor neuron fate by post-mitotic cells or the survival of these cells cannot be excluded.

Cyclopamine effects are probably specific, since PCR results indicated a selective and strong reduction in *patched1* and *olig2* mRNA expression after cyclopamine injection. Moreover, functional recovery and the number of axons that regenerated from the brainstem were not affected, suggesting the absence of global toxic effects of cyclopamine.

Why was behavior not influenced by cyclopamine treatment? Regenerated, ChAT<sup>+</sup> mature motor neurons partially replace the population of previously existing motor neurons that are lost around a spinal lesion site (Reimer et al., 2008). However, while motor neuron regeneration was reduced by 50%, an average of 377 new motor neurons remained, which is still in excess of the approximately 120 motor neurons that eventually show signs of full integration into the spinal network (Reimer et al., 2008). It is also possible that motor neuron regeneration around a spinal lesion site is not necessary for overall functional recovery. Interestingly, the fact that regeneration of axons from the brainstem was also unaffected, speaks against the idea that newly generated motor neurons exert a major trophic or tropic influence on these axons.

## Involvement of developmental signaling pathways in adult motor neuron regeneration

The remaining motor neuron generation after cyclopamine treatment may be attributable to other pathways that promote motor neuron regeneration. Our analysis indicates increased expression of *fgf3* and its target gene *spry4* (Fürthauer et al., 2001), as well as several retinoic acid related genes (Maden, 2006). These include the target gene *crabp2a*, which, for example, is active in motor neurons derived from embryonic stem cells in vitro (Chaerkady et al., 2009). Thus, our data is consistent with an involvement of additional embryonic signals, FGF and retinoic acid, in adult motor neuron regeneration. Together with the role of Shh and up-regulation of embryonic transcription factors Pax6, *olig2* and Nkx6.1 this suggests that embryonic signaling pathways are at least partially re-deployed during motor neuron regeneration. In contrast, during adult heart regeneration in zebrafish, transcription factors active during heart development (*nkx2.5*, *tbx5*) are not upregulated, whereas other genes (*msxB*, *msxC*, *deltaC*, *notch1b*) are expressed only in the regenerating heart (Raya et al., 2003).

## Is there a lesion-induced switch in pMN-like ependymo-radial glial cells for motor neuron generation?

Given that many pMN transcription factors and the *shha* signal are detectable at low levels also in the unlesioned spinal cord, one may speculate that a lesion simply augments a normal program of adult motor neuron generation. However, ventricular proliferation and motor neuron generation is extremely rare in the unlesioned spinal cord (one new BrdU<sup>+</sup> motor neuron in one fish was observed in a sample of 14 adult fish; Reimer et al., 2008). Only after a lesion, there is massive motor neuron generation (200 new BrdU<sup>+</sup> motor neurons within 1.5 mm around a spinal lesion site per fish). Moreover, genes necessary for hh pathway activity, *patched1* and *smoothed* (Chen et al., 2001) only become detectable in the lesioned spinal cord by in situ hybridization. Finally, a hh agonist alone failed to elicit motor neuron generation or ventricular proliferation in the unlesioned spinal cord (own unpublished observations). This suggests that additional lesion-induced changes are necessary to switch pMN-like ependymo-radial glial cells to motor neuron generation.

## pMN-like ependymo-radial glial cells may be multi-potent progenitor cells

pMN-like ependymo-radial glial cells are located in the *Tg(olig2:egfp)*<sup>+</sup> ventricular zone of the spinal cord and increase in number after a lesion. Likewise, the ventricular zone of *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells is enlarged after a lesion and these cells incorporate BrdU and are labeled by PCNA antibodies (Reimer et al., 2008). This suggests that the ependymo-radial glial cell populations expand after a lesion, presumably by symmetric cell divisions. This may result in the significant widening of the central canal during regeneration, also observed in other teleost species (Dervan and Roberts, 2003). We have shown previously by lineage tracing that *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial cells give rise to Hb9<sup>+</sup> motor neurons in the lesioned spinal cord (Reimer et al., 2008). It has been suggested that *Tg(olig2:egfp)*<sup>+</sup> ependymo-radial glial give rise to oligodendrocytes in the unlesioned spinal cord (Park et al., 2007). Thus pMN-like ependymo-radial glial cells are probably multi-potent progenitor cells, giving rise to themselves, motor neurons and oligodendrocytes. In contrast, mammalian forebrain ependymal cells, which produce glial cells and neuroblasts after a stroke lesion, fail to maintain their population by self-renewal (Carlen et al., 2009).

## Adult ependymo-radial glial cells may be the equivalent of embryonic neural tube progenitor cells

In the unlesioned adult spinal cord, we find expression of key defining factors of progenitor and signaling zones of the embryonic neural tube, *shha*, Pax6, Nkx6.1 and an *Tg(olig2:egfp)*

transgene in almost identical, partially overlapping relative positions around the central canal as in the developing neural tube (Varjosalo and Taipale, 2008). Similarly, the dorsal neural tube marker *pax7* is expressed in the dorsal spinal cord of the adult axolotl (Schnapp et al., 2005). Similar to progenitor cells in the developing neural tube, adult ependymo-radial glial cells contact the ventricle and the pial surface. For these reasons it is tempting to speculate that during the developmental thickening of the wall of the neural tube, these cells simply retain contact with the pial and ventricular surfaces together with their progenitor zone-specific expression profile of transcription factors. In such a scenario, the ventral *shha* expressing ependymo-radial glial cells would be cells that originally formed the floor plate of the embryonic neural tube.

### Do similar progenitor cells exist in the adult mammalian spinal cord?

It has been shown that stem cells exist in the adult mammalian spinal cord that can form neurospheres in vitro and neurons in vivo, when transplanted into a favorable environment (Shihabuddin et al., 2000). Notably, lineage tracing experiments indicate that ependymal cells are the main neurosphere forming cell population in the lesioned mouse spinal cord. These ependymal cells self-renew and generate scar-forming cells as well as oligodendrocytes after a lesion. They form part of the spinal cord ependyma and possess long radial processes, similar to ependymo-radial glial cells in zebrafish. However, processes do not reach the pial surface, but form endfeet on blood vessels (Meletis et al., 2008).

Our observations in adult fish raise the question whether adult mammalian spinal ependymal cells possess any dorso-ventral polarity, which might predispose them for the generation of specific neuronal cell types. *Pax6*, but not *Olig2* or *Nkx2.2*, becomes detectable in the ependyma of the injured rat spinal cord. However, it is unclear whether this expression is polarized (Yamamoto et al., 2001). One report (Chen et al., 2005) showed a surprisingly widespread increase in *shh* mRNA expression in the entire ependyma and several parenchymal cell types in mice. Interestingly, administering *Shh* or a *hh* agonist leads to ventricular proliferation and nestin expression in the lesioned spinal cord of adult rats (Bambakidis et al., 2003; Bambakidis et al., 2009), suggesting that mammalian spinal ependymal cells may also be capable of reacting to a *hh* signal. In the lesioned mammalian cortex astrocytes up-regulate *shh* expression, which regulates proliferation of *olig2*<sup>+</sup> precursor cells (Amankulor et al., 2009).

### Conclusions

Ependymo-radial glial cells in the spinal cord of adult zebrafish share morphological and molecular features with presumptive mammalian spinal stem cells. Our spinal lesion paradigm in zebrafish may thus be useful as a pharmacologically and genetically accessible model to determine the signals that allow motor neuron regeneration in the spinal cord of a fully adult vertebrate. *Shh* may be such a signal, as it is necessary for a pMN-like subpopulation of ependymo-radial glial cells to efficiently produce motor neurons in the lesioned spinal cord of adult zebrafish.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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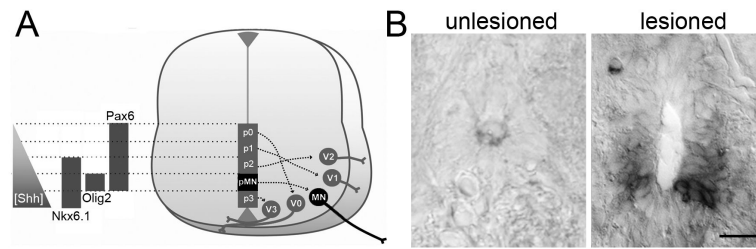
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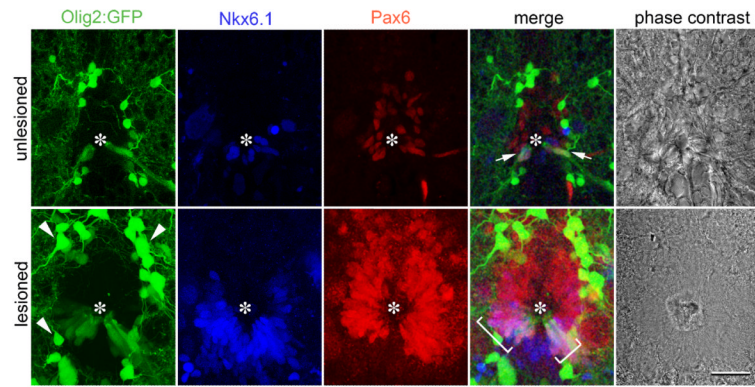


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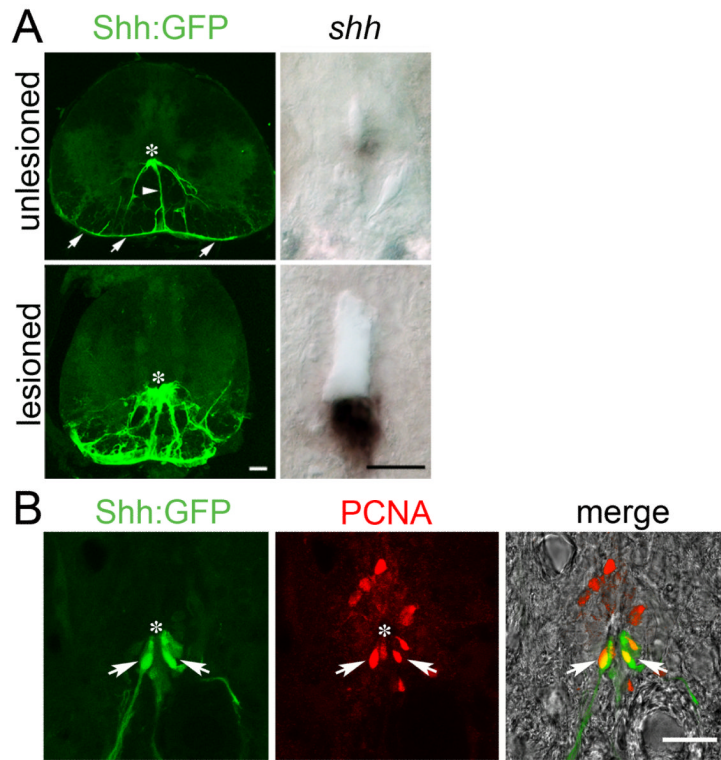


**Fig. 1.**

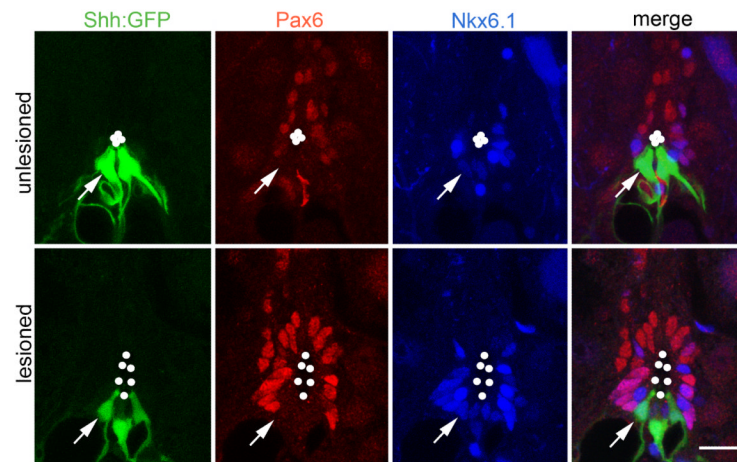
Expression of *olig2* mRNA is increased in the lesioned adult spinal cord close to the lesion site at 2 weeks post-lesion. **A:** Schematic presentation of transcription factor domains set up in the developing vertebrate neural tube by a ventro-dorsal gradient of Shh (modified after Vallstedt and Kullander, 2007). Different progenitor zones (p0-3) give rise to distinct classes of interneurons (V0-3). The motor neuron progenitor zone (pMN) gives rise to motor neurons (MN). **B:** Cross sections through the adult spinal cord are shown at the level of the central canal (dorsal is up). Ventricular cells in ventro-lateral positions around the central canal express detectable levels of *olig2* mRNA in the lesioned spinal cord. Bar = 25  $\mu$ m.



**Fig. 2.** Evidence for a pMN-equivalent zone in the ependymal layer of the adult spinal cord. Spinal cross sections at the level of the central canal (asterisks) are shown. In the unlesioned (arrows) and lesioned spinal cord (brackets) Pax6/*Tg(olig2:egfp)*/Nkx6.1 co-expressing ependymo-radial glial cells are present. Expression of all markers is increased close to a spinal lesion site at 2 weeks post-lesion. Arrowheads = oligodendrocytes; bar = 20  $\mu$ m.

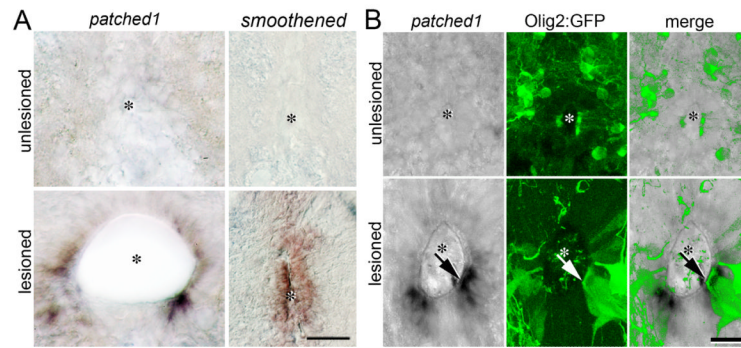


**Fig. 3.** Increased expression of *shha* and proliferation of  $Tg(shha:gfp)^+$  ependymo-radial glial cells in the ventricular zone of the lesioned spinal cord. Cross sections are shown of whole spinal cross sections ( $Tg(shha:gfp)$  in A only) or just for the area around in the ventricle at high magnification (all other images). Asterisks indicate the position of the ventricle. **A:** Expression of *shha* mRNA is increased in the ventral-most position of the ventricle and  $Tg(shha:gfp)^+$  ventral ependymo-radial glial cells are more numerous at 2 weeks post-lesion. **B:**  $Tg(shha:gfp)^+$  ependymo-radial glial cells actively proliferate at 2 weeks post-lesion, as indicated by double labeling with PCNA antibodies (arrows). Bars in A = 25  $\mu$ m, in B = 25  $\mu$ m.

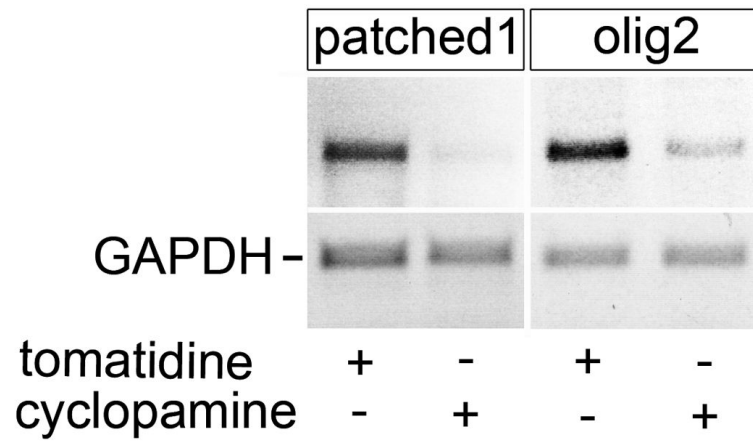


**Fig. 4.** *Tg(shha:gfp)*<sup>+</sup> ependymo-radial glial cells co-express Nkx6.1, but not Pax6. Spinal cross sections at the level of the central canal (outlined by dots) are shown. Arrows point to an individual *Tg(shha:gfp)*<sup>+</sup>/*Nkx6.1*<sup>+</sup>/*Pax6*<sup>-</sup> cell. Bar = 15  $\mu$ m.

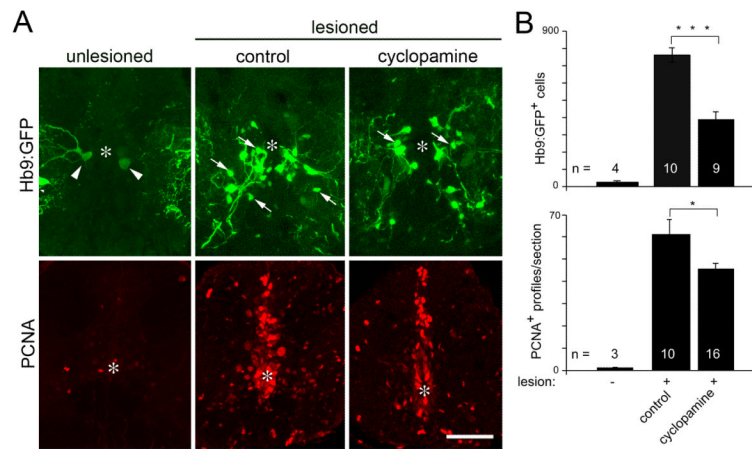




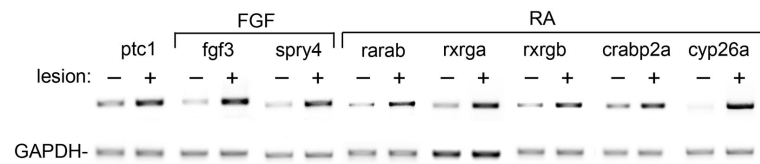
**Fig. 5.** Hh pathway genes are upregulated in ependymal cells of the lesioned spinal cord. Cross sections of the adult spinal cord at the level of the central canal (asterisk) are shown. **A:** Expression of *patched1* and *smoothened* mRNA is increased after a lesion. Note polarized (stronger ventral) expression of *patched1* mRNA, whereas *smoothened* mRNA is detectable along the dorso-ventral extent of the central canal of the lesioned spinal cord. **B:** In the lesioned, but not the unlesioned spinal cord, *Tg(olig2:egfp)<sup>+</sup>* ependymo-radial glial cells express detectable levels of mRNA for *patched1* (arrow), an indicator of Hh pathway activity. Bar in A = 25  $\mu\text{m}$ ; bar in B = 15  $\mu\text{m}$ .



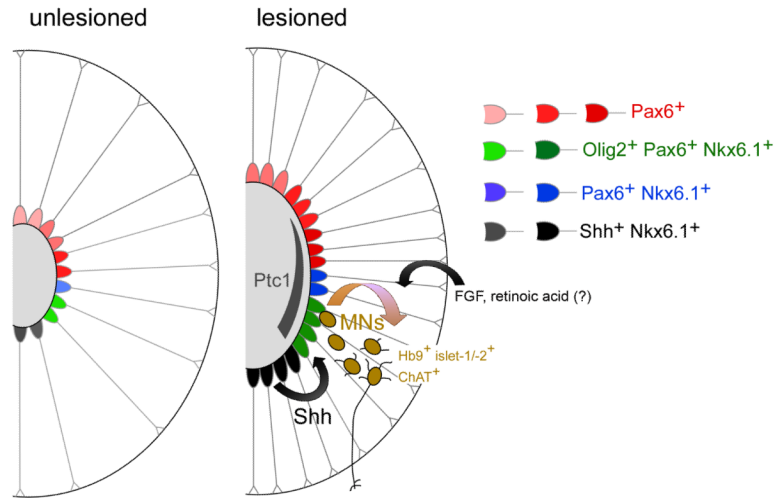
**Fig. 6.** Expression of *patched1* and *olig2* mRNA in the adult lesioned spinal cord is specifically reduced by intraperitoneal cyclopamine injection. PCR amplification of *patched1* and *olig2*, using GAPDH as a standard, is shown. Tomatidine injection is used as a negative control.



**Fig. 7.** Cyclopamine treatment impairs motor neuron regeneration. **A:** Spinal cross sections are shown. Asterisks indicate the central canal. Cyclopamine injection reduces the number of newly generated motor neurons (*Tg(hb9:gfp)*<sup>+</sup>; arrows) and proliferating cells in the ventricular zone (PCNA<sup>+</sup>). Arrowheads indicate large motor neurons in unlesioned animals. **B:** Quantification of reduced numbers of small, newly generated *Tg(hb9:gfp)*<sup>+</sup> motor neurons and PCNA<sup>+</sup> profiles depicted in A. Values for unlesioned animals are given for reference (Reimer et al., 2008). Control = injected with tomatidine. Bar = 50  $\mu$ m.



**Fig. 8.** FGF and retinoic acid signaling pathway related genes show increased expression in the lesioned adult spinal cord. PCR analyses of unlesioned and lesioned (14 days post-lesion) spinal cord, using GAPDH levels as a standard, are shown. All indicated genes show robust up-regulation in lesioned tissue. *Ptc1* as an indicator for Shh pathway activity is included as a positive control.



**Fig. 9.**

Summary of known events associated with motor neuron regeneration in adult zebrafish. Schematic cross sections through the spinal cord are shown (the central canal is represented by a grey oval). Different ependymo-radial glial cells in the unlesioned spinal cord express low levels (light shading) of different transcription factor combinations found in similar dorso-ventral positions in the embryonic neural tube. After a lesion, the central canal widens close to the lesion site, concomitant with an increase in numbers of ependymo-radial glial cells that also express higher levels of transcription factors (darker shading). A ventro-dorsal *hh* signal, evidenced by a ventro-dorsal *patched1* (*Ptc1*) mRNA expression gradient in ependymo-radial glial cells, contributes to production of Hb9<sup>+</sup>/islet-1/-2<sup>+</sup>/ChAT<sup>+</sup> motor neurons (MNs) from pMN-like ependymo-radial glial cells (green) after a lesion. Increased expression of genes related to FGF and retinoic acid signaling suggests involvement of these signals in motor neuron regeneration.