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## Lesion-induced generation of interneuron cell types in specific dorsoventral domains in the spinal cord of adult zebrafish

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TITLE

Lesion-induced generation of interneuron cell types in specific dorso-ventral

domains in the spinal cord of adult zebrafish

AUTHORS

Veronika Kuscha[1], Sarah L. Frazer[1], Tatyana B. Dias[1], Masahiko Hibi[2],

Thomas Becker[1]*, Catherina G. Becker[1]*§


ADDRESS

[1]Centre for Neuroregeneration, School of Biomedical Sciences, University of

Edinburgh, The Chancellor's Building, Edinburgh EH16 4SB, UK; e-mail:

thomas.becker@ed.ac.uk; catherina.becker@ed.ac.uk


[2]Bioscience and Biotechnology Center, Nagoya University Furo-cho, Chikusa-

ku Nagoya, Aichi, 464-8601 Japan


*Equal contributions
§for correspondence

RUNNING TITLE

Zebrafish spinal cord interneuron regeneration

ABSTRACT

In contrast to mammals, the lesioned adult spinal cord of zebrafish is capable of regenerating neurons. For example, motor neurons are generated from an olig2 expressing population of pMN-like ependymo-radial glial cells in a ventro-lateral position at the central canal. However, the extent of neuronal regeneration is unclear. Here we show, using a transgenic fish in which V2 interneurons are labeled by green fluorescent protein (GFP) under the control of the vsx1 promoter, that after a complete spinal cord transection, large numbers of V2 interneurons are generated in the vicinity of the lesion site. Tg(vsx1:GFP)[+] cells are not present in the unlesioned spinal cord and label with the proliferation marker BrdU after a lesion. Some medio-laterally elongated tg(vsx1:GFP)[+] cells contact the central canal in a medial position. These cells likely arise from a p2-like domain of ependymo-radial glial progenitor cells, indicated by co-expression of Pax6 and Nkx6.1, but not DsRed driven by the olig2 promoter, in these cells. We also present evidence that Pax2^{+} interneurons are newly generated after a spinal lesion, whereas the generation rate for a dorsal population of parvalbuminergic interneurons is comparatively low. Our results identify the regenerative potential of different interneuron types for the first time and support a model in which different progenitor cell domains in distinct dorso-ventral positions around the central

canal are activated by a lesion to give rise to diverse neuronal cell types in the adult zebrafish spinal cord.

INTRODUCTION

After a spinal lesion in mammals, progenitor cells proliferate but fail to produce neurons that could replace those that were lost due to the lesion (for recent review see Nogradi et al., 2011). Instead, large numbers of glial cells are produced that contribute to the glial scar (Barnabe-Heider et al., 2010). In contrast to mammals, the CNS of anamniotes (salamanders and fishes) functionally regenerates, there is little evidence for scar formation and neurons do regenerate (Becker and Becker, 2007; Ferretti, 2011; Sirbulescu and Zupanc, 2010). Specifically, in the lesioned adult spinal cord of zebrafish, there is no indication of a glial scar after complete spinal cord transection and motor neurons (Reimer et al., 2008) and serotonergic neurons (Kuscha et al., 2011) are newly generated. However, it is unknown whether all spinal cell types regenerate equally well and what the underlying cellular mechanisms are. Therefore, characterizing the progeny of proliferating progenitors in the adult spinal cord of zebrafish is an important aim.

Spinal cord transection triggers proliferation of cells mainly around the central canal in the vicinity of the lesion site. The proliferating cells are ependymo-radial glial cells, which contribute to the ependyma and reach the pia with a radial process (Reimer et al., 2008). Previous research has shown that ependymo-radial glial cells around the central canal in adult zebrafish differ in terms of their transcription factor expression profile. The most ventral cells express sonic hedgehog. Nkx6.1 immunoreactivity is found in slightly more dorsal cells. Ventro-lateral cells express olig2 and even more dorsal cells label with Pax6 in partially overlapping patterns (Reimer et al., 2009). This is reminiscent of the developing spinal cord, where olig2 expression demarcates

the motor neuron progenitor (pMN) domain, and nkx6.1 and pax6 co-expression in the absence of olig2 expression indicates the V2 interneuron progenitor (p2) domain just dorsal to the pMN domain (Briscoe and Novitch, 2008; Fuccillo et al., 2006; Jessell, 2000).

These similarities in transcription factor expression led us to hypothesize that ependymo-radial glial cells in the adult zebrafish spinal cord may be derived from embryonic neural tube progenitors and that these cells retained their dorso-ventral positional information (Reimer et al., 2009). If this was the case, different dorso-ventral domains should produce different neuronal cell types after a lesion. Indeed, in the adult lesioned spinal cord, motor neurons are regenerated from the olig2[+] domain, designated pMN-like domain, in a ventro-lateral position under the influence of a Hedgehog signal from ventral ependymo-radial cells (Reimer et al., 2009; Reimer et al., 2008). Serotonergic cells, which are produced in high numbers after a lesion, originate from the ventral, but not the dorsal central canal (Kuscha et al., 2011). It is not known which other neuronal cell types are generated in the lesioned spinal cord and where they originate.

Here we show that, as predicted from the embryonic pattern in vertebrates, undifferentiated V2 interneurons are generated in a domain of the ependymal layer, which co-labels with Pax6 and Nkx6.1 antibodies, and is just dorsal to the olig2 expressing domain. We therefore identify this domain as p2-like. Moreover, we show that other interneuron cell types show differences in regenerative capacity, indicating that regeneration of spinal cell types likely depends on the proliferative activity of specific progenitors.

MATERIALS AND METHODS

Animals

All fish are kept and bred in our laboratory fish facility according to standard methods (Westerfield, 1989) and all experimental procedures have been approved by the British Home Office. We used wild type (wik; ZFIN.org) and two transgenic lines. *Tg(olig2:DsRed2)* (Kucenas et al., 2008) labels oligodendrocytes and pMN-like ependymo-radial progenitor cells in a pattern that is indistinguishable from *tg(olig2:GFP)* (Shin et al., 2003). We have previously shown by in situ hybridization for *tg(olig2:GFP)* that the transgene accurately reports *olig2* expressing cells in the lesioned adult spinal cord (Reimer et al., 2009).

The *tg(vsx1:GFP)* transgenic line was generated using a bacterial artificial chromosome (zC67N1) containing the *vsx1* promoter (Kimura et al., 2008). Transgene expression specifically labels *vsx1* expressing cells in the embryonic (Kimura et al., 2008) spinal cord. In the adult lesioned spinal cord, trangene expressing cells also co-localize with the mRNA specifically in the medio-lateral aspect of the spinal cord (Fig. 1J-L). However, more cells were labeled wit the transgene than with the mRNA in this location, presumable due to greater stability of the transgene derived protein than the mRNA of the endogenous gene. Higher sensitivity of transgene detection than mRNA for endogenous genes has previously been reported (Reimer et al., 2008).

Spinal lesion and intrapertional injections of BrdU

Lesions were performed as previously described (Becker et al., 1997).

Fish were anesthetized in 0.033% aminobenzoic acid ethyl methyl ester (MS222; Sigma). A longitudinal incision was made at the side of the fish to expose the vertebral column. The spinal cord was completely transected 3.5 mm caudal to the brainstem-spinal cord junction under visual control. Bromodeoxyuridine (BrdU, B9285, Sigma) was injected intraperitoneally at a concentration of 5 mg/ml in a volume of 25 µl PBS (pH 7.4) at 0, 2 and 4 days post-lesion (dpl), as described (Reimer et al., 2008). Analysis took place at 2 or at 6 weeks post-lesion (wpl).

Antibody characterization

Please see table 1 for all antibodies used. The anti-BrdU ascites labeled cell nuclei in the lesioned spinal cord only after prior incubation of the fish with BrdU. Thus, it does not cross-react non-specifically with other antigens in the tissue of interest (Kuscha et al., 2011).

The anti-GFP serum labels different cellular structures only in GFP transgenic animals, demonstrating specific detection of the antigen.

The Islet-1/2 antibody specifically labels motor neurons in the embryonic spinal cord of zebrafish. These cells can be co-labeled by an HB9 transgene, visualizing the entire morphology of motor neurons including their peripheral axon, the defining feature of motor neurons (data not shown). Moreover, the antibody labels *tg(HB9:GFP)*^{+} motor neurons after spinal cord lesion in adult fish (Reimer et al., 2008). The Islet-1/2 antibody labeled cell nuclei in CHO cells transfected with *islet-1*, but not in untransfected cells (Thor et al., 1991).

The HB9 (also known as MNR-2) antibody reveals a pattern in the lesioned zebrafish spinal cord that is very similar to that of islet-1/2 (Reimer et al., 2008) and also labels *tg(HB9:GFP)*^{+} motor neurons in embryonic and adult zebrafish (Reimer et al., 2008). Mis-expression of MNR-2 using a replication competent retroviral vector, results in an ectopic expression pattern labeled with the HB9 antibody in the chick neural tube, but not in control vector injected animals (Tanabe et al., 1998).

The pattern labeled by the Pax2 antibody closely resembles the mRNA expression patterns of *pax2a* and *pax2b* detected by in situ hybridization in the embryonic spinal cord of zebrafish (Batista and Lewis, 2008). In the adult spinal cord of zebrafish, it labels cells that express GFP under the *pax2a* promoter, indicating that *pax2a* expressing cells are specifically recognized by this antibody (data not shown). The Pax2 antiserum labels bands of the expected size of 46kDa for Pax2b and 48kDa for Pax2a in Western blots of embryonic mouse kidney and human Wilm's tumors (Dressler and Douglass, 1992).

The Parvalbumin7 antibody, generated against the zebrafish antigen, detects Purkinje cells in the cerebellum that are also labeled by in situ hybridization for the gene (Bae et al., 2009), indicating specificity of the antibody.

The Pax6 antibody labels two bands of about 50 kDa in Western blots of tissue from the adult mouse brain, eye, and olfactory bulb, but not liver. The two closely migrating bands result from alternative splicing (Davis and Reed, 1996; Walther and Gruss, 1991). In adult zebrafish, the antibody labels ventro-dorsal ependymo-radial glial cells, in a pattern that is highly

reminiscent of the developing neural tube. This pattern has been confirmed with an independently raised antibody to Pax6 (Reimer et al., 2008). In the zebrafish retina, the Pax6 antibody labels specifically cells in the inner nuclear layer and the ganglion cell layer (Jaszai et al., 2011).

The Nkx6.1monoclonal antibody labeled the expected 36-kDa band in Western blots of a purified GST-Nkx6.1 fusion protein, but not the related GST-Nkx6.2 protein in mouse (Pedersen et al., 2006). Moreover, this antibody labeled the expected Nkx6.1 double band of 44 and 46 kDa in protein extracts from the SV40 large T-antigen transformed insulinoma βTC-3 cell line (Pedersen et al., 2006).


Immunohistochemical procedures

Immunohistochemistry on vibrating blade microtome sections, 50 μm in thickness, has been described (Reimer et al., 2008). Deeply anesthetized (0.1% MS222 for more than 5 minutes) fish were transcardially perfused with 4% paraformaldehyde (PFA) in PBS and post-fixed in PFA overnight. If not stated otherwise, washing steps were for 5 minutes at room temperature. After transcardiac perfusion, spinal cords or brains were embedded in agar (4% in PBS), cut and sections were collected in 24-well plates in PBS.

For labeling of Parvalbumin7, HB9 and Islet-1/2, antigen retrieval was performed by incubating sections at 80°C in citrate buffer (10mM sodium citrate in PBS, pH 6.0) for 30 minutes. After washing in PBS/0.1% Triton-X 100 (PBStx-0.1), sections were incubated in 50mM glycine in PBStx-0.1 for 10 minutes followed by washing in PBStx-0.1 for 15 minutes. Sections were blocked in normal goat serum for 30 minutes. Incubation with one or two

different primary antibodies in PBStx-0.1 was performed overnight at 4°C. Subsequently, sections were incubated with secondary antibodies (diluted 1:200; secondary Cy2-, Cy3- and Cy5-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Finally, the sections were mounted in 90% glycerol/PBS. Specific binding of the secondary antibody was controlled for by omitting the primary antibody on alternating sections.

To detect BrdU, sections were washed in PBS/0.5% Triton-X-100 (PBStx-0.5) for 1 h at RT, 3x in PBS and 1x with HCl 37%, diluted 1:8 in deionized water. Then, sections were incubated in the diluted HCl at 37°C in a water bath for 20 minutes. After washing 6 x in PBS, sections were incubated in 50 mM glycine in PBS/0.3% Triton X-100 (PBStx-0.3) for 10 minutes followed by another 15 minutes wash in PBStx-0.3. From here on blocking and antibody incubations were performed as described above, except for using PBStx-0.3 instead of PBStx-0.1. For documentation and analysis, we used either a Zeiss LSM 510 or a Zeiss 750 LSM Confocal Microscope using 20x and 63x oil immersion lenses.

In situ hybridization

The method for non-radioactive in situ hybridization on vibrating blade microtome sections (50 µm thickness) has been described (Lieberoth et al., 2003). The plasmid to generate a probe for *vsx1* (Kimura et al., 2008) was generously provided by Dr. Shin-ichi Higashijima. The plasmid was digested with XhoI and the mRNA was transcribed using the T3 promoter.

Solutions were made up in RNAse free water. If not stated otherwise,

washing steps were 5 minutes long. Sections of paraformaldehyde-fixed

tissue were transferred into 24-well plates, washed twice in PBS/0.1% Tween-

20 (PBST-0.1) and then digested with proteinase K (stock concentration:

Roche PCR Grade, 0.3 µg/ml in 0.1 M Tris pH 8 and 0.05 M EDTA; working

solution: 0.7µl/ml) for 9 minutes at room temperature. After washing twice with

glycine in PBST-0.1 (2mg/ml), the tissue was re-fixed in 4%

paraformaldehyde for 20 minutes. After washing 4 x in PBST-0.1, supernatant

was removed and sections were washed once in 300 µl hybridization buffer [5

ml formamide, 2.5 ml 20x SSC, 10 µl Tween, 100 µl yeast tRNA (100 mg/ml),

2.38 ml DEPC-H2O, 10 µl heparin (50 mg/ml)]. After replacing this with fresh

hybridization buffer (500µl/well), plates were wrapped with parafilm and kept

at 55°C for at least 3 hours. Digoxigenin (DIG)-labeled probes (1:1000 to

1:500 in hybridization buffer) were incubated at 80°C for 10 minutes, briefly

centrifuged and chilled on ice for 2 minutes. Hybridization buffer in the wells

was replaced with probe solution. Plates were sealed with parafilm and

incubated at 55°C overnight. The following incubations were done at 55°C.

The solutions were preheated. Sections were washed twice in 50%

formamide/2xSSC + 0.1% Tween-20 for 30 minutes each, once in 1x SSC +

0.1% Tween-20 for 15 minutes and twice in 0.2 x SSC + 0.1% Tween-20 for

30 minutes each. Then, the sections were blocked in 10% Roche blocking

reagent (10 g blocking reagent in 100 ml PBST-0.1) at room temperature.

Anti-DIG alkaline phosphatase-coupled fab fragments (Boehringer/Roche)

were diluted 1:2000 in blocking reagent, added to the sections and left

overnight at 4°C. Sections were washed 6 times for 20 minutes each in PBST-

0.1 on a shaker and then once in PBS. Staining solution was prepared by

adding one 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium tablet (Sigma) to 10 ml de-ionised water. After washing once in staining solution, sections were incubated in fresh staining solution for 30 minutes to overnight, depending on the kinetics of signal development. Finally, sections were washed several times in PBS and mounted in 70% glycerol.

### Cell quantification

If not stated otherwise, numbers of cells were determined from stereological counts of confocal images of three randomly selected sections (50 µm in thickness) from the spinal cord in the region within 750 µm rostral to the lesion site and three sections from within 750 µm caudal to the lesion site. Cell numbers were calculated for the entire 1.5 mm surrounding the lesion site.

### Statistical analysis

For statistical analysis, the program Prism (GraphPad Software, La Jolla, CA, USA) was used. Variability of values is given as SEM. Statistical significance was determined using the Mann-Whitney-U test for single comparisons or ANOVA followed by Dunnet's or Tukey's post test for multiple comparisons.

RESULTS

Our goal was to determine the regenerative potential of interneuron cell types in the lesioned adult spinal cord of zebrafish.

### _Tg(vsx1:GFP)_[±] cells are newly generated after a spinal lesion

During development, _vsx1_ mRNA and the _tg(vsx1:GFP)_ transgene are early markers for undifferentiated V2 interneurons that develop in the p2 domain adjacent to the motor neuron progenitor domain in zebrafish (Batista et al., 2008; Kimura et al., 2008). We decided to use the transgenic line _tg(vsx1:GFP)_ (Kimura et al., 2008), to determine whether a spinal lesion triggers generation of such interneurons in adults.

In unlesioned _tg(vsx1:GFP)_ fish, very few _tg(vsx1:GFP)_[+] cells were observed in 1500 µm of spinal cord at a midthoracic level (Fig. 1A) and no mRNA expression was observed for the gene by in situ hybridization (data not shown). This was expected, because during development expression of the transgene and the endogenous gene is transient in V2 interneurons (Kimura et al., 2008) and hardly any new neurons are generated in the unlesioned adult spinal cord (Reimer et al., 2008). After a lesion of the adult spinal cord, the number of _tg(vsx1:GFP)_[+] cells was significantly increased at 2 wpl (24-fold) and at 6 wpl (22-fold) compared to unlesioned control fish (unlesioned: 26.5 ± 19.08 cells/1500 µm, n = 3; 2 wpl: 628.2 ± 80.9 cells/1500 µm, n = 12; 6 wpl: 573.0 ± 162.94 cells/1500 µm, n = 6; ANOVA, p = 0.0213; Fig. 1 A-E).

_Tg(vsx1:GFP)_[+] cells were located in a slightly ventro-lateral position of the central canal (Fig. 1B-D). Some of the _tg(vsx1:GFP)_[+] cells contacted the

central canal. They were medio-laterally elongated and some of them had extended processes (Fig. 1D). In situ hybridization of *vsx1* mRNA in transgenic fish showed upregulation of the endogenous gene from undetectable levels at 2 wpl, overlapping with labeling of the transgene (Fig. 1J-L).

To directly show that *tg(vsx1:GFP)*^{+} cells were newly generated after lesion, we injected BrdU at 0, 2 and 4 days after lesion. At 2 wpl, 20.4% (6.3 ± 1.55 of 29.3 ± 4.64 cells, n = 4) of the *tg(vsx1:GFP)*^{+} cells were labeled with BrdU (Fig. 1F-I). Not all *tg(vsx1:GFP)*^{+} cells were double-labeled with BrdU even though these cells were almost completely absent in unlesioned animals. This low labeling rate was expected, because BrdU has limited bioavailability and the same injection scheme also labels only ~25% of the newly generated *Tg(HB9:GFP)*^{+} neurons after a lesion (Reimer et al., 2008). Thus, BrdU labeling shows that *tg(vsx1:GFP)*^{+} cells are newly generated after a lesion

To confirm that *tg(vsx1:GFP)*^{+} cells were indeed distinct from newly generated motor neurons, which appear in a similar location during regeneration and are of similar size (Reimer et al., 2008), we performed immuno-histochemical labeling of HB9 (Fig. 2A-C) and Islet-1/2 (Fig. 2D-F) in spinal cross sections of *tg(vsx1:GFP)* fish at 2 wpl. The vast majority of *tg(vsx1:GFP)*^{+} cells did not co-label with HB9 (92%; n = 110 cells in 10 sections of 3 animals) or Islet-1/-2 (96%; n = 86 cells in 12 sections of 2 animals) antibodies. Moreover, most *tg(vsx1:GFP)*^{+} cells did not co-label (98%; n = 158 cells in 9 sections of 3 animals, Fig. 2G-I) with an antibody to Pax2, a transcription factor expressed in a subpopulation of interneurons,

including V1 interneurons (Burrill et al., 1997). This demonstrates that *tg(vsx1:GFP)*^{+} cells are a population of newly-generated neurons that is distinct from motor neurons and Pax2^{+} interneurons in the lesioned adult spinal cord.

### *Tg(vsx1:GFP)*[±] cells emerge from a p2-like progenitor domain

In the developing neural tube, V2 interneurons emerge from the *pax6*[+]/*nkx6.1*[+]/*olig2*[-] domain, dorsal to and contiguous with the *olig2*[+] motor neuron progenitor domain (Jessell, 2000; Wilson and Maden, 2005). We hypothesized that we would find a similar pattern during regeneration, since ependymo-radial glial cells, the progenitor cells in the adult spinal cord, show a similar dorso-ventral polarity of transcription factor domains as the developing neural tube. In fact, Nkx6.1, Pax6, and the *tg(olig2:GFP)* transgene are strongly upregulated after a spinal lesion in their specific domains (Reimer et al., 2009).

Assuming that *tg(vsx1:GFP)*^{+} cells with ventricular contact had been born most recently, we analyzed their dorso-ventral marker expression as evidence of their domain of origin. A graphical summary of transcription factor expression domains in the developing neural tube that also applies to the adult central canal is given in Figure 3A. *Olig2* expression indicates the embryonic and adult motor neuron progenitor domain and cells in this domain are double-labeled for the motor neuron marker HB9 after a lesion (Reimer et al., 2009). To determine whether *tg(vsx1:GFP)*^{+} cells might emerge from this domain, we crossed *tg(vsx1:GFP)* with *tg(olig2:DsRed2)* transgenic fish. Of 18 *tg(vsx1:GFP)*^{+} cells with central canal contact, only one *tg(vsx1:GFP)*[+] cell

co-labeled with the *tg(olig2:DsRed2)* transgene (n = 4 animals; Fig. 3K-M).

The remainder of the cells did not double label with *tg(olig2:DsRed2)* and

were found slightly dorsal to the *tg(olig2:DsRed2)*[+] domain of spinal progenitor

cells. Therefore, 95% of the *tg(vsx1:GFP)* transgene expressing cells were

negative for the *tg(olig2:DsRed2)* transgene and did not emerge from the

motor neuron progenitor domain.

      Next we determined immunolabeling of Pax6 (dorsal transcription

factor) and Nkx6.1 (ventral transcription factor) in *tg(vsx1:GFP)* animals at 2

wpl. Both of these factors, but not *olig2*, are present in the embryonic p2

domain (Fig. 3A). Of 12 ventricle-contacting *tg(vsx1:GFP)*^{+} cells (n = 2

animals), half (50.0%) only expressed Pax6, one third (33.3%) were

Pax6[+]/Nkx6.1[+] and 17% co-localized with neither Pax6 nor Nkx6.1 labeling

(Fig. 3B-J). Importantly, no cells were only labeled by Nkx6.1[+], which would

have indicated ventral positional identity of the cells. *Tg(vsx1:GFP)*^{+} cells

without contact to the central canal, but adjacent to it, showed similar ratios.

Of 64 *tg(vsx1:GFP)*^{+} cells analyzed (n = 2 animals), half (50.0%) were

pax6[+]/nkx6.1[-], approximately one third (34.4%) were Pax6^{+}/Nkx6.1[+], and a

small minority (3%) were Pax6^{-}/Nkx6.1[+]. Cells that co-labeled with neither

Pax6 nor Nkx6.1 amounted to 12.5%. (Fig. 3K-M). This data suggests that

many *tg(vsx1:GFP)*[+] were generated from a Pax6^{+}/Nkx6.1[+]/*tg(olig2:DsRed)*[-]

p2-like domain during spinal cord regeneration in adult zebrafish. The

observation that 50% of cells with central canal contact were Pax6^{+}/Nkx6.1[-],

suggests that Nkx6.1 is quickly down-regulated in differentiating

*tg(vsx1:GFP)*^{+} cells or that some *tg(vsx1:GFP)*^{+} cells are generated from an

even more dorsal Pax6^{+}/Nkx6.1[-] domain.

<u>Pax2$^{\pm}$ cells are newly generated after lesion</u>

As established above, Pax2$^+$ neurons are a population of interneurons that is distinct from that of *tg(vsx1:GFP)*$^+$ interneurons. In contrast to *tg(vsx1:GFP)*$^+$ cells, Pax2$^+$ cells are present in the unlesioned spinal cord (Fig. 4A). After a lesion, cell numbers remained unchanged at 2 and 6 wpl, compared to unlesioned animals (unlesioned: 2661.1 ± 392.67 cells/1500 µm, n = 4; 2 wpl: 2608.4 ± 56.25 cells/1500 µm, n = 4; 6 wpl: 2048.6 ± 371.81 cells/1500 µm, n = 4; Fig. 4B,C).

Next we determined BrdU labeling in Pax2$^+$ cells. Since Pax2 is an antigen present in differentiated neurons, as it is seen in unlesioned animals, we determined double-labeling with BrdU at 6 wpl after injection at day 0, day 2 and day 4 post-lesion, such as to give potential newly-generated cells sufficient time for differentiation. For example, newly-generated differentiated ChAT$^+$ motor neurons were only observed at 6 wpl, but not at 2 wpl, when the number of undifferentiated *tg(HB9:GFP)*$^+$ motor neurons peaked (Reimer, 2008). At 6 wpl, 17.0% of all Pax2$^+$ cells were labeled with BrdU (348.6 ± 172.34 of 2048.6 ± 371.81 Pax2$^+$ cells, n = 4, Fig. 4D-G). This relatively high percentage of labeled cells was surprising, given that cell numbers did not show significant changes after a lesion. It is therefore likely, that newly-generated Pax2$^+$ cells replaced cells that were lost as a consequence of the lesion.

<u>Dorsal Parvalbumin7$^{\pm}$ cells are rarely newly generated after a lesion</u>

To our knowledge, parvalbuminergic cells have not been described in the adult zebrafish spinal cord. We found small Parvalbumin7$^+$ cells with a diameter of 5 - 7 µm in the dorsal and ventral horn and larger cells (diameter 12 - 17 µm) only in the ventral horn in adult zebrafish spinal cord. The large cells were reminiscent of motor neurons. Indeed, analysis of 30 large Parvalbumin7$^+$ cells and 49 small Parvalbumin7$^+$ in the ventral horn of spinal cross section of transgenic *tg(HB9:GFP)* fish (n = 3 animals), revealed that 80% (24 cells) of the large cells were *tg(HB9:GFP)*$^+$ (Fig. 5A-G). In contrast, only 2% (1 cell) of the small Parvalbumin7$^+$ cells in the ventral horn co-localized with GFP in *tg(HB9:GFP)* transgenic fish. This indicates that some motor neurons in the ventral horn are Parvalbumin7$^+$.

Since the small cells in the dorsal horn presented as a homogeneous population that has not previously been analyzed, we focused our post-lesion analysis on these. Numbers of Parvalbumin7$^+$ cells in the dorsal horn did not change after a lesion for up to 6 wpl (unlesioned: 638.9 ± 92.24 cells/1500 µm, n = 4; 2 wpl: 735.2 ± 76.68; n = 6; 6 wpl: 716.7 ± 185.39, n = 4, Fig. 5H,I). After BrdU injection at day 0, day 2 and day 4 post-lesion, we observed 0.3% (1.9 ± 1.85 cells/1500 µm, n = 6) BrdU labeled Parvalbumin7$^+$ cells at 2 wpl and 2% (16.7 ± 5.55 cells, n = 4) Parvalbumin7$^+$/BrdU$^+$ cells at 6 wpl (Fig. 5J-M). Thus, dorsal parvalbuminergic cells are added at a much lower rate than Pax2$^+$ cells at 6 wpl, as only 2% of the parvalbuminergic neurons could be labeled with BrdU, but 17% of the Pax2$^+$ cells (p = 0.03, Mann-Whitney U-test), suggesting differences in lesion-induced neurogenesis for these cell types.

DISCUSSION

We demonstrate for the first time a p2-like progenitor domain in the adult zebrafish spinal cord and show that the lesioned spinal cord of adult zebrafish is capable regenerating different types of interneurons to different extents (summarized in Fig. 6).

### *Tg(vsx1:GFP)*[±] cells are newly generated after a lesion and originate from a p2-like domain of ependymo-radial glial cells.

*Tg(vsx1:GFP)*[+] interneurons are not observed in the unlesioned adult spinal cord. They appear in large numbers after a lesion, concentrated in a medio-lateral area. Co-labeling of these cells with the proliferation marker BrdU after a lesion supports that they were newly generated after a lesion. During development, *vsx1* expressing cells differentiate into two types of V2 interneurons (Kimura et al., 2008). *Tg(vsx1:GFP)*^{+} cells in the regenerating adult spinal cord probably down-regulate expression of the transgene and the endogenous mRNA upon differentiation, suggested by the lack of labeling of transgene and mRNA in the unlesioned adult spinal cord and similar to the sequence of their differentiation during early embryonic development (Kimura et al., 2008). Therefore, determining the full sequence of differentiation of *tg(vsx1:GFP)*^{+} cells has to await lineage tracing techniques which are becoming available in adult zebrafish (Rothenaigner et al., 2011).

At 2 to 6 wpl, many *tg(vsx1:GFP)*[+] interneurons were still in contact with the ventricle, thereby indicating their likely place of origin. These cells were found just dorsal of the *olig2:DsRed* expressing domain. Co-labeling with Pax6 and Nkx6.1 antibodies allowed us to determine a p2-like identity for

the domain from which these cells originated, as p2 cells are $nkx6.1^+$, $pax6^+$ and $olig2^-$ during development (Lee and Pfaff, 2003). However, a large number of $tg(vsx1:GFP)^+$ interneurons were labeled only by Pax6 antibodies, suggesting rapid down-regulation of $nkx6.1$ during differentiation of these cells.

Previously, we observed that $HB9^+$ motor neurons originated from $tg(olig2:GFP)^+$ ependymo-radial progenitors, just ventral to where $tg(vsx1:GFP)^+$ interneurons were observed in contact with the central canal. Thus, the progenitor domains of motor neurons and V2 interneurons have the same spatial relationship during regeneration as during development. Serotonergic interneurons that can also be seen to emerge from the ependymal layer have a broader ventral domain of origin, with the $olig2$ expressing domain forming its dorsal boundary (Kuscha et al., 2011). This strongly suggests that the transcription factor combination expressed in different dorso-ventral progenitor cell domains in the adult spinal cord determines which neuronal cell types may be generated from specific progenitor cells after injury.

### Are there differences in the regenerative capacity and mechanisms for different neuronal cell types?

In contrast to $tg(vsx1:GFP)^+$ interneurons, $Pax2^+$ cells were present in the parenchyma in the unlesioned and lesioned spinal cord, making Pax2 a marker for differentiated neurons. This prevented us from making any observations as to the origin of these cells. The same was true for parvalbuminergic neurons. However, it was striking to find that a substantial

number of Pax2^{+} neurons were added after a spinal lesion, as indicated by BrdU labeling, whereas small parvalbuminergic neurons in the dorsal horn were rarely newly generated. It will be interesting to determine whether different progenitor cells in the spinal cord have different capacities to produce specific neuronal cell types after a lesion, or whether there are feedback mechanisms from differentiated neurons to progenitor cells, regulating their proliferation. For instance, in the salamander, dopamine is a negative signal for the generation of dopaminergic neurons and ablation of dopaminergic cells or pharmacological inhibition of dopamine triggers generation of new dopaminergic cells from ependymo-radial glial cells (Berg et al., 2011). Thus, as cell numbers of parvalbuminergic cells in the lesioned adult spinal cord of zebrafish do not change, suggesting that hardly any were lost after a lesion, their progenitor cells might not have gotten the signal to proliferate. In contrast to a feedback model of neurogenesis, an over-production of serotonergic neurons of more than 5 times more cells than in unlesioned animals has been observed after a spinal lesion in adult zebrafish (Kuscha et al., 2011). This excess is later reduced again. Similarly, many more *tg(HB9:GFP)*^{+} motor neurons are generated than are later integrated into the spinal circuitry as mature ChAT^{+} motor neurons (Reimer et al., 2008). These observations speak against a tightly regulated feedback mechanism of neuronal regeneration in the spinal cord.

Interestingly, generation of immature *tg(HB9:GFP)*^{+} motor neurons peaked at 2 wpl and sharply declined towards 6 wpl (Reimer et al., 2008). In contrast, production of immature *tg(vsx1:GFP)*[+] V2 interneurons continued at 6 wpl, indicating that the duration of proliferative activity may also differ for

different types of progenitor cells. Overall, these data suggest that during cellular regeneration in the CNS of anamniotes, different feedback-dependent and -independent mechanisms may occur to generate new neurons from specific progenitor cell populations.

<u>Are dorsal signals and progenitor zones lost in the adult spinal cord?</u>

So far, marked regeneration of ventral (serotonergic) to ventro-medial (motor neurons, V2 interneurons) cell types has been demonstrated, whereas more dorsal parvalbuminergic interneurons show a lower rate of regeneration. During adult regeneration, Sonic hedgehog derived from ventral ependymo-radial glial cells promotes regeneration of serotonergic and motor neurons (Kuscha et al., 2011; Reimer et al., 2009). This is a recapitulation of the developmental hedgehog signal from the floor plate (Briscoe and Novitch, 2008; Fuccillo et al., 2006; Jessell, 2000). In contrast, neurogenesis in the dorsal spinal cord is regulated by bone morphogenetic protein (BMP) and Wnt signaling during development (Muroyama et al., 2002; Nguyen et al., 2000). However, in the lesioned adult spinal cord we have not been able to find any indication of dorsal signaling centers that may promote regeneration of dorsal cell types, using in situ hybridization for *BMP2* and *BMP4*, the *wnt*-related genes *tcf7* (Bonner et al., 2008) and *wnt3a* (Megason and McMahon, 2002; Muroyama et al., 2002), and the dorsal markers *pax3* and *pax7* (Mansouri and Gruss, 1998) (unpublished observations). It has been suggested that the central canal in amniotes consists only of cells derived from the ventral neural tube, as the dorsal parts of the neural tube are lost due to closure and fusion (Fu et al., 2003). If the central canal in zebrafish is formed in a similar way,

dorsal progenitor zones could be lost, preventing regeneration of dorsal cell types, such as dorsal parvalbuminergic cells. However, in adult salamanders *pax7* is expressed in the dorsal spinal cord (Schnapp et al., 2005).

Interestingly, in mammals, adult ependymal cells express Nkx6.1 (Fu et al., 2003) and after a lesion upregulate *pax6* expression (Yamamoto et al., 2001). This would give all ventricular cells a p2-like identity, yet no neurogenesis occurs from these cells. Instead, their lesion-induced proliferation contributes cells to the glial scar (Meletis et al., 2008). Future research will have to determine whether the dorsal spinal cord in zebrafish indeed lacks signals and progenitor zones that may promote regeneration of dorsal cell types.

Cytoarchitecture of the adult spinal cord

Relatively little is known about the cytoarchitecture of the adult zebrafish spinal cord and our markers have revealed some interneuron population in specific locations. For example, the Parvalbumin7 antibody identifies different classes of neurons in the dorsal and ventral horn, judged by different locations, sizes and marker expression of the cells. While the exact morphology and function of these cell types remains to be elucidated, we found Parvalbumin7-positive motor neurons, as indicated by co-labeling in very large *tg(HB9:GFP)*^{+} neurons. Interestingly, such motor neurons also exist in mammals and have been described as particularly resilient against degeneration (Elliott and Snider, 1995).

In contrast to adults, in the embryonic spinal cord of zebrafish a number of different interneuron cell types have been described and their

functions are being elucidated (Gabriel et al., 2011; McLean et al., 2007; Wyart et al., 2009). It will be a future challenge to compare the functional organization of the adult with that of the developing spinal cord in order to be able to fully compare this regenerating system with the perhaps similarly organized, but non-regenerating mammalian spinal cord.

<u>Conclusion</u>

Our results show that interneurons are generated in the lesioned spinal cord of an adult vertebrate and emphasize the importance of ependymo-radial glial cells in the ventricular zone for cellular regeneration after a spinal lesion. In mammals, cells with radial processes that have endfeet on blood vessels are also present in that zone and show lesion-induced proliferation, but fail to generate neurons (Meletis et al., 2008). Our results show that such cells retain dorso-ventral positional identity in an adult vertebrate, which predisposes these progenitor cells to generate specific neuronal cell types.

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TABLES

Table 1 Information on antibodies used in this study

| Antigen | Immunogen | Manufacturer (species antibody was raised in, mono- vs. polyclonal, catalog or lot number) | Diluted |
|---------|-----------|-----------|---------|
| Bromodeoxyuridine | BrdU | Serotec (Munich, Germany), rat, monoclonal, clone BU1/75 (ICR1)OBT0030CX | 1:500 |
| GABA | Aminobutyric acid (GABA) conjugated to BSA | Sigma-Aldrich, rabbit, polyclonal, A 2052 | 1:1000 |
| GFP | Recombinant full length protein | Abcam (Cambridge, UK), chicken, polyclonal, ab13970 | 1:500 |
| HB9 | KLH-conjugated chick HB9 NH2 peptide (MEKSKNFRIDALLA) | Developmental Studies Hybridoma Bank (DSHB, Oregon, USA), | 1:1000 |

| | | mouse, monoclonal, MNR2, 81.5C10-a | |
|---|---|---|---|
| Islet-1 homeobox | Synthetic peptide corresponding to rat Islet-1 amino acids 178–349 | DSHB, mouse, monoclonal, 40.2 D6 | 1:1000 |
| Nkx6.1 | Glutathione S-transferase fusion protein protein containing amino acids 299-365 of Nkx6.1 of rat | DHSB, mouse, monoclonal, F55A10 | 1:1000 |
| Parvalbumin7 | Glutathione S-transferase fusion protein containing amino acids (aa) 1–109 of parvalbumin7 of zebrafish | Masahiko Hibi (Riken Center for Developmental Biology Kobe, Japan), mouse, monoclonal | 1:1000 |
| Pax2 | Recombinant protein containing 22kD of the Pax2 sequence corresponding to amino acids 188-385 | Covance (Princeton, USA), rabbit, polyclonal, PRB-276P | 1:300 |

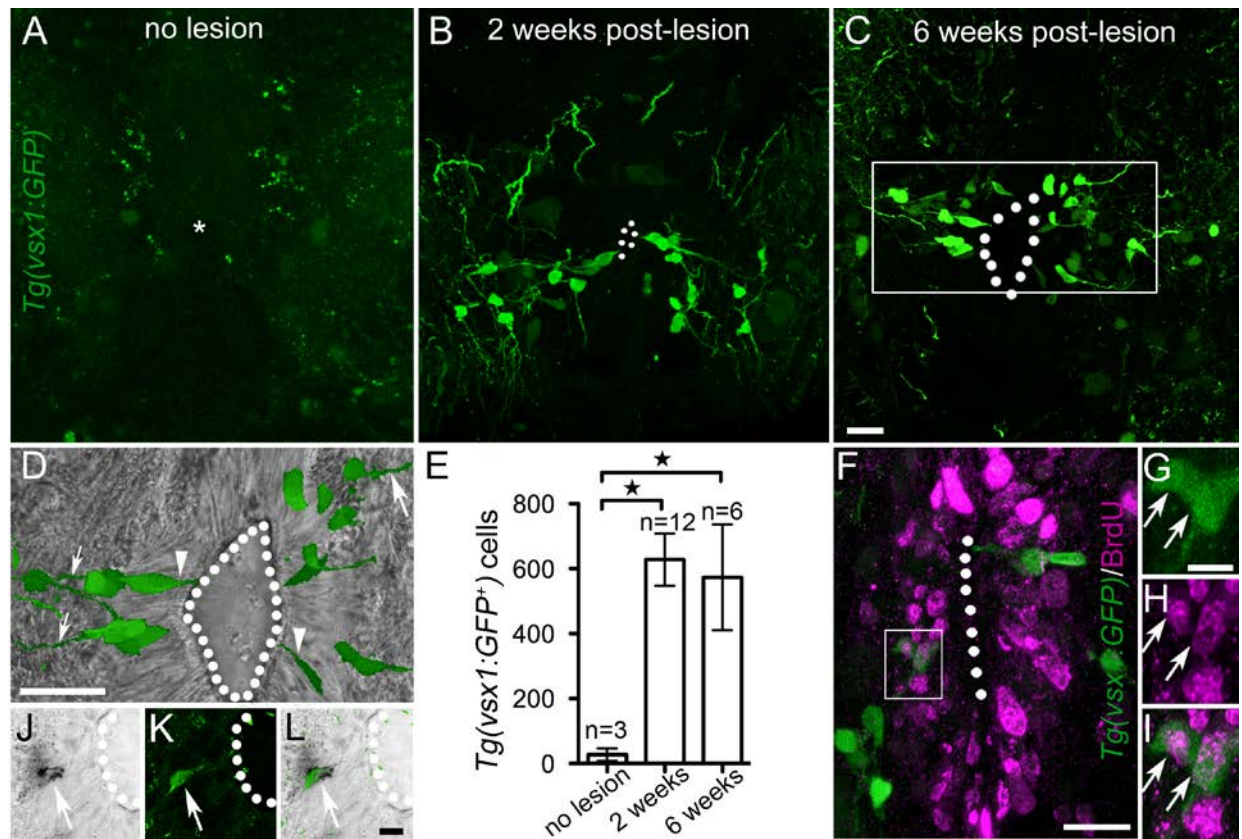| Pax6 | C-terminus of the mouse Pax6 protein (QVPGSEPDMSQYW PRLQ) | Covance, rabbit, polyclonal, PRB-278P | 1:300 |

FIGURES:



Fig. 1: *Tg(vsx1:GFP)*^{+} cells are mainly found after a lesion and are newly

generated. GFP labeling in spinal cross sections of *tg(vsx1:GFP)*[+] fish is

shown; dorsal is up; asterisk and dots indicate position of the central canal. **A:**

*Tg(vsx1:GFP)*^{+} cells are rarely observed in unlesioned animals. **B-D:** At two

(B) and six wpl (C), numerous GFP^{+} cells are observed in a medial position.

The boxed area in C is shown in higher magnification in D with fluorescent

cells overlayed on phase-contrast illumination to identify the central canal.

Arrowheads point to cell bodies with contact to the central canal and arrows

point to extending neurites. **E:** Numbers of *tg(vsx1:GFP)*^{+} cells are

significantly increased at 2 and 6 wpl. **F-I:** Nuclei of *tg(vsx1:GFP)*^{+} cells

(green) are labeled with BrdU (mangenta) at two wpl. The boxed area in F is

shown in higher magnification in G-I. Arrows point to double-labeled cells. **J-**

**L:** *Vsx1* mRNA (J) and *tg(vsx1:GFP)* transgene (K) co-localize in the overlay

(L) at 2 wpl. *P < 0.05. Scale bar in C = 15 µm for A-C, bar in D = 15 µm.

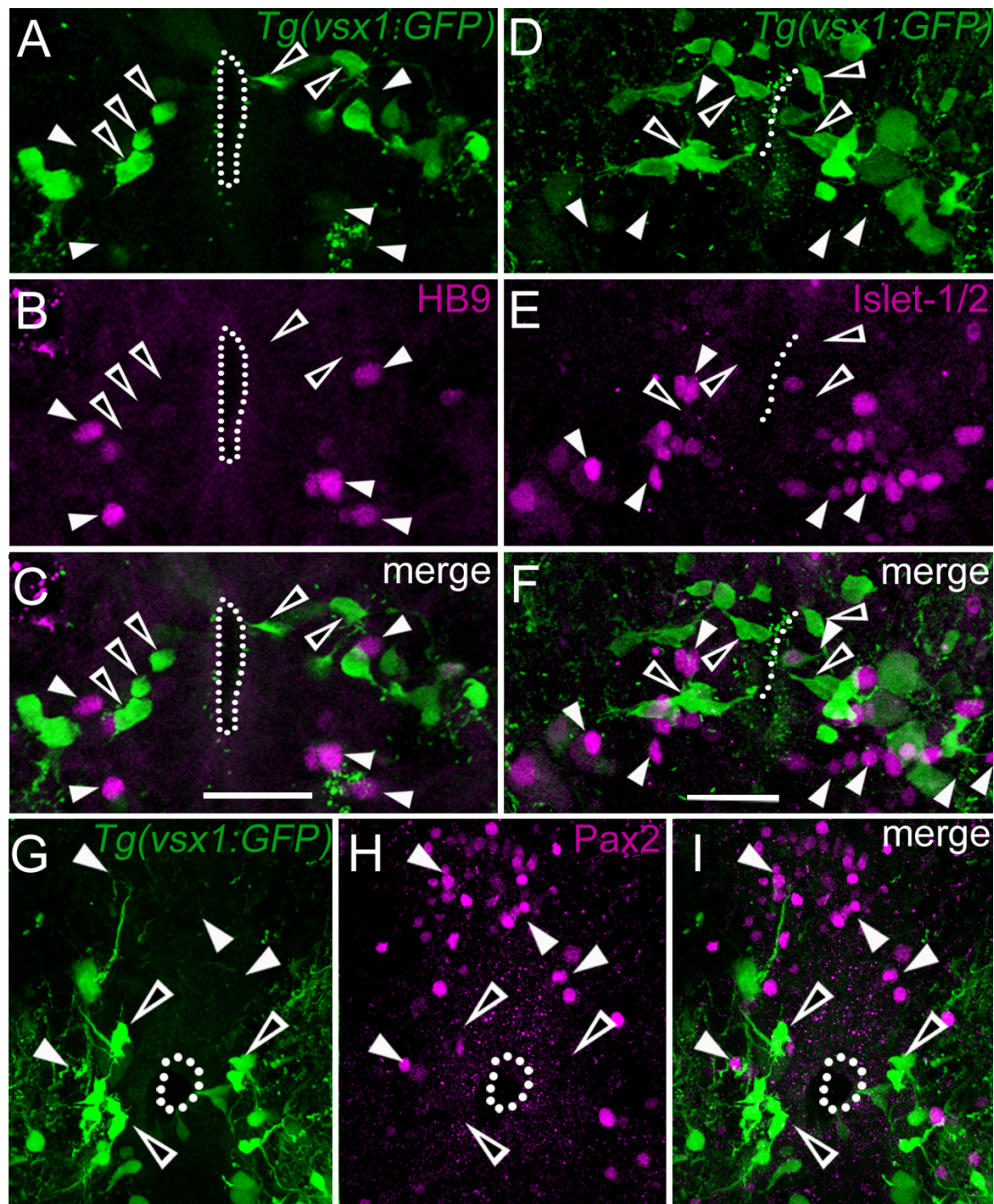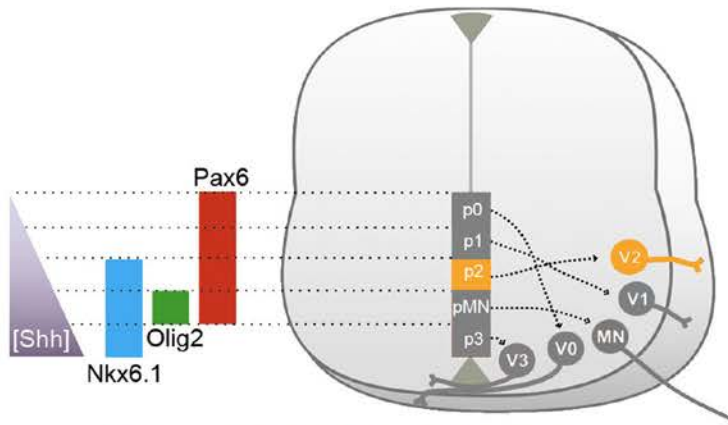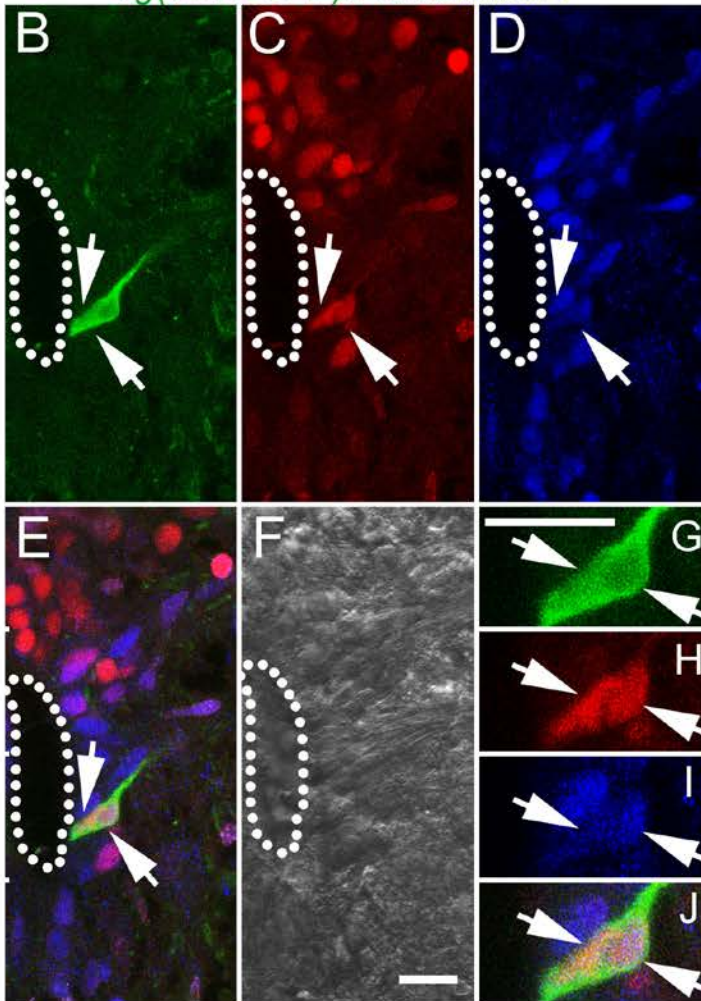Scale bar in F = 15 µm, bar in G = 10 µm for G-I, bar in L = 25 µm for J-L.

Fig. 2: *Tg(vsx1:GFP)*^{+} cells are distinct from HB9^{+}, islet-1/2[+], and Pax2^{+} cells. Spinal cross sections are shown; dorsal is up; central canal is outlined by dots. **A-C:** HB9 immuno-labeled cells (solid arrowheads) do not overlap with *tg(vsx1:GFP)*^{+} cells (empty arrowheads). **D-F:** Islet-1/2 immuno-labeled cells (solid arrowheads) do not overlap with *tg(vsx1:GFP)*^{+} cells (empty arrowheads). **G-I:** Pax2 immuno-labeled cells (solid arrowheads) are distinct

from *tg(vsx1:GFP)*^{+} cells (empty arrowheads). Scale bars in C = 25 µm for A-C, bar in F = 25 µm for D-F, bar in I = 10 µm for G-I.

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**A**

Pax6

[Shh]

Nkx6.1

Olig2

p0
p1
p2
pMN
p3

V2
V1
V3 V0 MN

*Tg(vsx1:GFP)*/Pax6/Nkx6.1

B C D

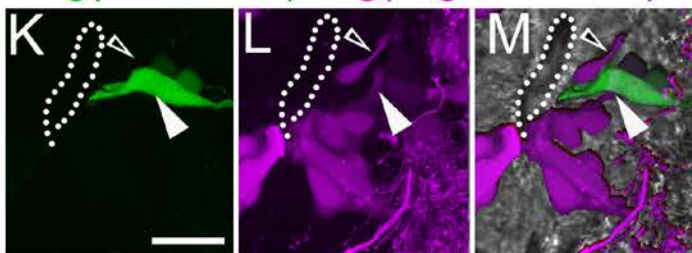E F G

H

I

J

*Tg(vsx1:GFP)*X*Tg(olig2:DsRed2)*

K L M

Fig. 3: *Tg(vsx1:GFP)*^{+} cells originate from a p2-like ventricular domain. Spinal cross sections are shown; dorsal is up; central canal is outlined by dots. **A:** Schematic presentation of transcription factor domains set up in the developing vertebrate neural tube by a ventro-dorsal gradient of Sonic hedgehog (Shh) (modified after Vallstedt and Kullander, 2007). The expression domains of the indicated transcription factors are similar in the ventricular zone of the adult central canal. The p2 domain (Pax6^{+}/Nkx6.1[+]/Olig2^{-}) gives rise to V2 interneurons in the developing spinal cord. **B-F**: Triple labeling of *tg(vsx1:GFP)*, Pax6 and Nkx6.1 shows two adjacent *tg(vsx1:GFP)*^{+} cells that are Pax6^{+} and Nkx6.1[+] (arrows) at 2 wpl, **G-J:** A higher magnification of the triple-labeled cells is shown. **K-M**: Solid arrowheads indicate two *tg(vsx1:GFP)*^{+} cells that are located dorsal of the *Tg(olig2:DsRed2)* transgene expressing domain in double transgenic fish. The empty arrowheads point to a *tg(olig2:DsRed2)*[+] cell without ventricular contact that may be an oligodendrocyte or neuron. The dotted line indicates the central canal. Scale bars in F = 10 μm for B-F, bar in G = 10 μm for G-J, bar in K = 15 μm for K-M.
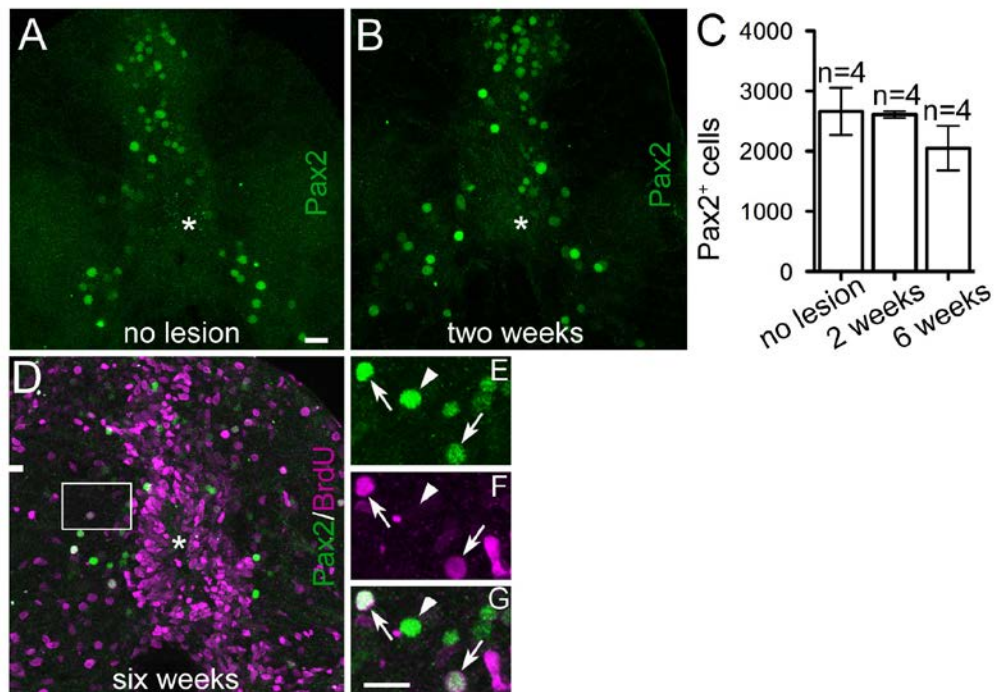
Fig. 4: Pax2$^+$ cells are generated after a spinal lesion. Spinal cross sections are shown; dorsal is up, central canal is indicated by asterisks. **A,B**: Pax2$^+$ cells are present in the parenchyma of the unlesioned (A) and lesioned (B) spinal cord at 2 wpl. **C:** Numbers of Pax2$^+$ cells in unlesioned animals did not significantly differ from those in animals at 2 and 6 wpl. **D-G:** Labeling of Pax2 (green) and BrdU (mangenta) shows double-labeled cells at 6 wpl. The boxed area in D is shown in higher magnification in E (Pax2), F (BrdU) and G (merge). Arrows point out double-labeled cells; arrowhead indicates a cell only labeled by Pax2 antibodies. Scale bar in A = 15 µm for A, B and D, in G = 5 µm for E, F and G**.**
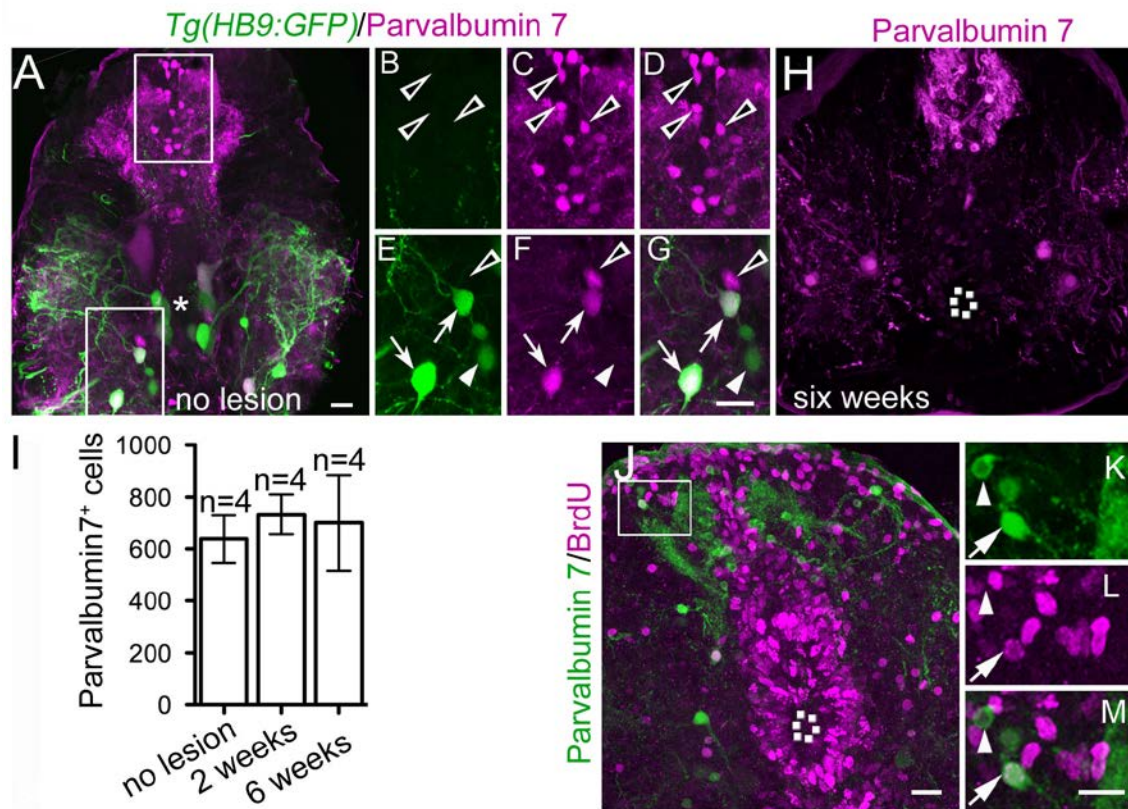
Fig. 5: Parvalbumin7^{+} cells are rarely generated after a spinal lesion. Spinal cross sections are shown; dorsal is up, central canal is indicated by asterisks or dots. **A-G**: Labeling of Parvalbumin7 in a spinal cross section of an unlesioned transgenic *tg(HB9:GFP)* (green) fish is shown. Small Parvalbumin7^{+} cells boxed in the dorsal horn and Parvalbumin7^{+} cells boxed in the ventral horn in A are shown in higher magnification in B-D, and E-G, respectively. Empty arrowheads point to Parvalbumin7^{+} only cells, filled arrowheads point to *tg(HB9:GFP)[+]* only cells and arrows indicate double-labeled cells. **H**: Labeling of Parvalbumin7 in a spinal cross section at 6 wpl is shown. **I**: Numbers of Parvalbumin7^{+} cells did not significantly differ between unlesioned controls and fish at 2 and 6 wpl. **J-M***: Double-labeling of Parvalbumin7 and BrdU in spinal cross sections at 6 wpl is shown. The boxed area in J is shown in higher magnification in K (Parvalbumin), L (BrdU) and M (merge). The arrowhead points to a cell only labeled by Parvalbumin7

antibodies and arrows point to a double-labeled cell. Scale bar in A = 20 μm

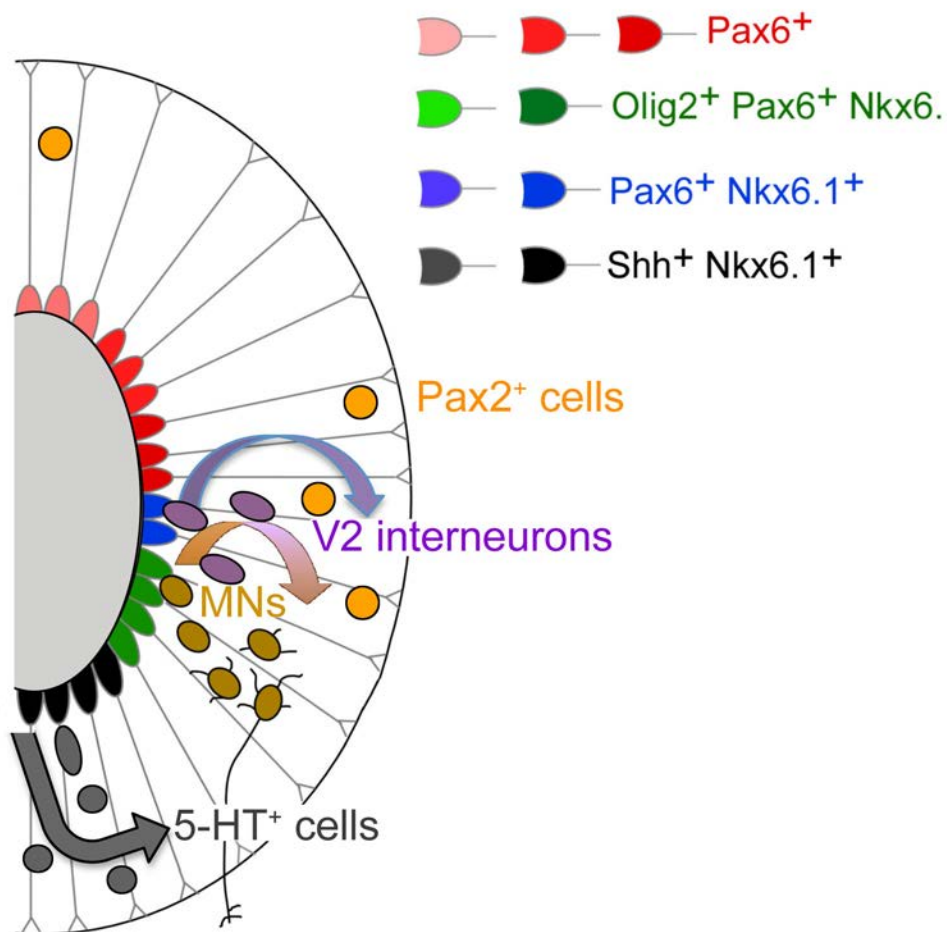for A and H, in G = 20 μm for B-G, in J = 20 μm, in M = 10 μm for K-M.

Fig. 6: Summary of progenitors zones and newly generated neuronal cell types in the adult lesioned spinal cord. Schematic cross section through the spinal cord is shown (the central canal is represented by a grey oval). Serotonergic neurons have a ventral origin (black and green progenitor cells; Kuscha et al., 2011). Motor neurons originate in the pMN-like zone (green; Reimer et al., 2008). V2 cells originate in the p2-like domain (blue; this report). The origin of newly generated Pax2+ interneurons remains to be elucidated.