Cellular Response to Spinal Cord Injury in Regenerative and Non-Regenerative Stages in *Xenopus Laevis*

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Abstract

**Background** The efficient regenerative abilities at larvae stages followed by a non-regenerative response after metamorphosis in froglets makes *Xenopus* an ideal model organism to understand the cellular responses leading to spinal cord regeneration.

**Methods** We compared the cellular response to spinal cord injury between the regenerative and non-regenerative stages of *Xenopus laevis*. For this analysis, we used electron microscopy, immunofluorescence and histological staining of the extracellular matrix. We generated two transgenic lines: i) the reporter line with the zebrafish GFAP regulatory regions driving the expression of EGFP, and ii) a cell specific inducible ablation line with the same GFAP regulatory regions. In addition, we used FACS to isolate EGFP+ cells for RNAseq analysis.

**Results** In regenerative stage animals, spinal cord regeneration triggers a rapid sealing of the injured stumps, followed by proliferation of cells lining the central canal, and formation of rosette-like structures in the ablation gap. In addition, the central canal is filled by cells with similar morphology to the cells lining the central canal, neurons, axons, and even synaptic structures. Regeneration is almost completed after 20 days post injury. In non-regenerative stage animals, mostly damaged tissue was observed, without clear closure of the stumps. The ablation gap was filled with fibroblast-like cells, and deposition of extracellular matrix components. No reconstruction of the spinal cord was observed even after 40 days post injury. Cellular markers analysis confirmed these histological differences, a transient increase of vimentin, fibronectin and collagen was detected in regenerative stages, contrary to a sustained accumulation of most of these markers, including chondroitin sulfate proteoglycans in the NR-stage.

The zebrafish GFAP transgenic line was validated, and we have demonstrated that is a very reliable and new tool to study the role of neural stem progenitor cells (NSPC). RNASeq of GFAP-EGFP cells allowed a clear demonstration that indeed these cells are NSPC. On the contrary, the GFAP-EGFP transgene is mainly expressed in astrocytes in non-regenerative stages. During regenerative stages, spinal cord injury activates proliferation of NSPC, and we found that are mainly fated to form neurons and glial cells. Specific ablation of these cells abolished proper regeneration, confirming that NSPC cells are necessary for functional regeneration of the spinal cord.

**Conclusions** The cellular response to spinal cord injury in regenerative and non-regenerative stages is profoundly different between both stages. A key hallmark of the regenerative response is the activation of NSPC, which massively proliferate to reconstitute the spinal cord, and are differentiated into neurons. Also very notably, no glial scar formation is observed in regenerative stages, but a transient, glial scar-like structure is formed in non-regenerative stage animals.

Introduction

Anatomical and functional regeneration of the spinal cord (SC) varies among the animal kingdom. While jawed and teleost fishes, urodele amphibians including salamanders and triton, lampreys and reptilians
such as turtles, have very efficient regenerative capabilities [1–4]. Other species such as anuran amphibians, including *Xenopus laevis* (*X. laevis*) are able to regenerate the SC at larval stages, but this capacity is lost during metamorphosis [5]. On the contrary, mammals are not able to attain efficient regeneration after SC injury (SCI) producing long-lasting effects [6]. Comparing the cellular response to injury in regenerative and non-regenerative model organisms is important to understand which cells facilitate or impede spinal cord regeneration.

The mammalian adult SC central canal (CC) mainly contains ependymal cells astrocytes [7] and some cerebral fluid contacting neurons [8]. The presence of neural stem progenitor cells (NSPC) has been demonstrated by *in vitro* experiments based on their ability to form neurospheres [9], and are mainly derived from cells expressing FoxJ1, Nestin or Glial fibrillary acidic protein (GFAP) [10–12]. NSPCs form neurons *in vitro* or when transplanted into a neurogenic environment, but not in the SC [13]. In rodents, spinal cord injury (SCI) generates more neurospheres, and nestin expression levels are increased, suggesting an activation of NSPC [12, 14–15]. Despite this, most activated NSPC cells are fated to astrocytes, and formation of new neurons is not observed *in vivo* [10]. In spite of neurosphere formation, *in vivo* proliferation of NSPC is rare, and just a very low proliferative capacity is present in dorsal and ventral uni- and Bi-ciliated ependymal cells [16].

Furthermore, following SCI a fibrotic and glial scar in the lesion site is formed by microglia, astrocytes, inflammatory cells, meningeal fibroblasts and pericytes together with an abundant extracellular matrix (ECM) containing chondroitin sulfate proteoglycans (CSPGs) and collagen [17–18]. The main function of this scar is to contain the inflammatory response, protecting the spinal cord from further damage [19–21]. In addition, the scar blocks axonal growth, inhibiting spinal cord regeneration [22]. Although, recent work challenged this dogma and disproved that astrocytes were the main CSPGs producers, instead, astrocytes secretes neuronal growth factors essential for neuronal survival, but, other cell types produce CSPGs which lead to the lack of regeneration [23–24].

A very different cellular response is observed in regenerative model organisms. In zebrafish and salamanders, the CC of the SC is mainly composed of ependymo-radial glia (ERG) cells. These cells are reminiscent of embryonic radial-glial, and express GFAP and Sox2, both markers of NSPC [25–27]. ERG have different functions after SCI, including a role as neural progenitors, and the generation of a permissive substrate for axonal regeneration [27]. In both, zebrafish and salamanders, injury activates massive proliferation of ERG cells, and expression of Sox2 is required for activation of cell division [28–29]. In fish, ERG cells are neural progenitors and can generate motoneurons and interneurons in response to injury [30–34]. Cell fate experiments showed that most of the new neurons are derived from a population of GFAP⁺ ERG cells [35].

In addition, ERG can also provide a permissive environment for axon growth in the adult fish. Processes from GFAP⁺ ERG cells elongate into the injury site connecting the two ends of the injured spinal cord making a “glial bridge” that could provide a permissive substrate for axon regeneration [35–38]. Interestingly, in zebrafish larvae, axons grow into the injury site before the glial bridge is formed.
suggesting that at this stage an “axonal bridging” between the two spinal cord stumps is established [27, 39].

*X. laevis* provides a unique experimental paradigm to compare regenerative and non-regenerative responses in the same specie [5, 40]. Pre-metamorphosis stages (NF stage 48–54) show a very efficient SC regeneration and are considered regenerative stages (R-stage). This ability is lost during metamorphosis (NF stage 66), and post-metamorphic animals including froglets are unable to regenerate the SC therefore are denominated as non-regenerative stages (NR-stages) [41–47]. At R-stages, most cells lining the CC have a radial glial morphology, are uniciliated, and express Sox2 [48]. While in NR-Stages, most cells lining the CC are multiciliated with an advance maturation and differentiation state and only few cells are uniciliated [48]. In R-stages, but not in the NR-stages, SCI induces a massive and transient proliferation of Sox2/3+ progenitor that is required for proper spinal cord regeneration, and formation of new neurons [46–47]. In R-stages, glial cells closely associated with growing axons in the ablation gap, suggesting a possible role for them in generating a glial bridge to aid in axonal regeneration [42]. Little evidence of glial scar formation in non-regenerative stages of *Xenopus* has been reported, so far, scar tissue was found encapsulating the end of the spinal cord lesion in post-metamorphic frogs [44].

Here, we compare the cellular response to SCI of the SC central canal, between the R- and NR-stages of *Xenopus laevis*. By using electron microscopy, we found a very different cellular response between both stages. In R-stage animals, the spinal cord and CC organization were rapidly restored. During regeneration, cells lining the CC seal the injured stumps already at 2 days after injury, activating a proliferative response followed by formation of rosette-like structures in the ablation gap. The CC was filled with cellular material including cells with similar morphology to the cells lining the central canal, neurons, axon, and even synaptic structures. 20 days after transection, the spinal cord was almost regenerated. A very different response was detected in NR-stage animals, mostly damaged tissue was observed during the first week, with no clear closure of the stumps. The ablation gap was filled with fibroblast-like cells, and deposition of ECM components, and no reconstruction of the spinal cord was observed. These differences in histological response were confirmed by cellular markers analysis. In R-stage animals, a transient increase of vimentin, fibronectin and ECM material was detected, contrary to a sustained accumulation of most of these markers, and CSPG, in the NR-stage.

For a more detailed study of the cellular response, we prepared a transgenic line using the zebrafish GFAP regulatory regions to drive EGFP expression. Characterization of this transgenic line showed expression in radial glial cells in R-stages, and astrocytes in NR-stage froglets. RNAseq analysis of the cells expressing the transgene in R-stage, demonstrated that they correspond to NSPC. At the R-stage spinal cord, injury activates proliferation of NSPC that are fated to form neurons. Ablation of these cells abolishes proper regeneration, confirming that are necessary for a functional regeneration of the spinal cord at NF stage 50.

**Results**
Cellular response to injury in regenerative and non-regenerative stages

The cellular organization of the spinal cord CC in *Xenopus laevis* changes between regenerative and non-regenerative stages [48]. To determine the cellular response to spinal cord injury between regenerative (R-stages, NF stage 50) and non-regenerative (NR-stages, NF stage 66) stages, we performed a detailed cellular analysis.

The spinal cord of R-stage animals was injured by full transection as described previously [40] (Fig. 1A), and tissues were analyzed by light and electron microscopy at different days post transection (dpt). At 2 dpt (Fig. 1B,D), a complete sealing of the rostral stump was observed (Fig. 1B, arrowheads in Fig. 1D). The cells lining the CC close to the injury site were not affected by the lesion. To identify ultrastructural changes in CC cells after SCI, we analyzed ultrathin sections. Cells lining the CC, characterized in the control as type I, II or III [48], lack junction complexes compared to controls (Fig. 1E, arrowheads), contain swelled mitochondria in their apical pole (Fig. 1E, arrow), and frequent centriolar satellites were found (see supplementary material, Fig. S1A, arrowheads). As expected, we identified abundant cells showing mitotic figures indicating cell division [46–47], almost half of the cellular clusters undergoing cell division have no contact with the central canal lumen (Fig. 1F), while the other half are in direct contact with it (Fig. 1G). Although in a lower proportion, cell division in the CC has been also observed in uninjured animals [47–48]. Conspicuous among the cells lining the CC was the presence of donut- and phone-like shaped mitochondria, phenome not observed in control animals (see supplementary material, Fig. S1B, arrowheads). Of note, we found cells in the CC that were being extruded toward the lumen (Fig. 1B,H arrowheads). Regarding infiltration in the injury site we found very few red blood cells (data not shown), abundant macrophages (Fig. 1C, arrowheads) with an electron dense cytoplasm (Fig. 1I), and a high number of ribosomes (data not shown) that are phagocytosing cellular debris (Fig. 1I), and some neutrophils (see supplementary material, Fig. S1C) that were not observed in controls.

At 6 dpt cells accumulate in the lumen of the central canal (Fig. 1J,K). These cells showed a higher nucleus/cytoplasm ratio similar to the cells lining the CC, a lax chromatin, a scarce cytoplasm with few organelles, and the absence of cellular junctions between them or with cells of the spinal cord (Fig. 1K, arrows). Interestingly, some cellular expansions within the lumen exhibited a high density of light vesicles, and few small dense core vesicles (Fig. 1L, white arrowheads) in near proximity to structures reminiscent of postsynaptic densities (Fig. 1L, black arrowheads). At this time, clusters of 20–24 cells forming rosette like structures were found in the ablation gap (Fig. 1J, M). These cells are very similar to those found in the uninjured central canal; resembling type III cells previously described [48]. These cells have a characteristic neuroepithelium organization; with a basal lamina containing collagen (see supplementary material, Fig. S1D), a high nucleus/cytoplasm ratio, apical mitochondria, abundant apical interdigitations with adherent cell junctions (see supplementary material, Fig. S1E), a high content of intermediate filaments (see supplementary material, Fig. S1F), and the presence of a cilium (data not shown).
At 10 dpt some continuity between the rostral and caudal stumps was observed (Fig. 1N). Surprisingly, abundant bundles of axons were found inside the lumen of the caudal central canal (Fig. 1N, O). These bundles were mainly composed by unmyelinated axons (Fig. 1P, orange) that were in close contact with synaptic vesicles (Fig. 1P, arrowheads), but also some myelinated axons were detected (Fig. 1R, arrowhead). The axon bundles were surrounded by cells with a fusiform nucleus that have a lax chromatin (Fig. 1O, arrowheads), and desmosome-like junctions were found between those cells (Fig. 1Q). These cells in close contact with axons have a condensed chromatin resembling a neuronal morphology (Fig. 1S). In line with the observation at 6 dpt, rosettes and some cells are still present in the ablation gap (data not shown), and the central canal, respectively.

Finally, as described before [47] at 20 dpt we observed an almost complete reconstruction of the spinal cord (Fig. 1T). However, the continuity of the central canal was not perfect (Fig. 1T, arrowhead). Importantly, the cells lining the central canal recover its normal organization as a pseudo-stratified epithelium (Fig. 1U), with apical mitochondria, microvilli (Fig. 1V), and desmosome cell junctions (Fig. 1W). Few cells remained inside the lumen of the central canal (Fig. 1U, red line).

A similar analysis was carried out in NR-stage animals. For this the spinal cord of animals at stage 66 was transected as described [40] (Fig. 2A), and the CC was analyzed at different times after injury. Contrary to the response observed in the R-stage, at 2 dpt the cells lining the CC were severely damaged, with the presence of many empty spaces between cells, leading to a strong disorganization and loss of the pseudo-stratified epithelial organization (Fig. 2B,C, arrowheads). An important loss of intracellular organelles is observed in the remaining ependymal cells (data not shown). In addition, we observed a massive invasion of the ablation gap with blood cells, (Fig. 2B,D; red shadow), and the deposition of extracellular matrix (ECM) components (Fig. 2D, arrowheads). A massive disorganization of the central canal, is still observed at 6 dpt, together with a sustained increase in the extracellular spaces between cells, and the presence of vacuolated cells (Fig. 2E,F; see arrowheads). In addition, a massive presence of red blood cells, macrophages (Fig. 2E,G), and microglia (data not shown) was still detected in the injury site.

At 10 dpt, the CC cells have recovered some epithelial organization (Fig. 2H,I), and have an abundant number of mitochondria in the apical surface (Fig. 2I, J) which resemble the lateral ependymal cells described before [48]. Unlike the response at the R-stage, froglets at the NR-stage are characterized by the absence of proliferation and rosette-like structures. Ten days after injury the borders of the rostral and caudal stump were surrounded by glial processes (Fig. 2K, white arrowheads), containing abundant intermediate filaments (Fig. 2L, white arrowheads). Finally, at 20 dpt the presence of red blood cells, and immune cells in the injury site had decreased (Fig. 2M), and the ablation gap is mainly filled by fibroblast-like cells, characterized by long nuclei (Fig. 2N, white arrowhead), a very dilated rough endoplasmic reticulum (Fig. 2O, white arrowheads), and an ECM containing abundant collagen fibers (Fig. 2P). These morphological features correlate with the complete lack of swimming capacities at 20 dpt in NR-stage [46–47].
In summary, R-stages revealed a dynamic regenerative process characterized by a fast response of cells lining the central canal to rapidly seal the injured stumps, activating a proliferative response followed by formation of rosette-like structures in the ablation gap. In addition, cells are extruded into the lumen of the central canal, which is also filled with axons and synaptic vesicles. Regenerated spinal cords, although not always with a perfect morphology, indicates an efficient resolution of the regeneration at 20 dpt. On the contrary, in the NR-stage, cells lining the central canal are deeply affected after injury, and instead, red blood cells and macrophages populate the injury site. After several days, cells lining the canal recover their ultrastructure, but the injury site is filled with glial cells processes, fibroblast and collagen fibers, reminiscent of the glial scar described in mammals, and a proper recovery of the spinal cord was not observed.

### Analysis of the presence of glial scar markers in response to spinal cord injury

One of the hallmarks of the cellular response to SCI in mammals is the formation of a glial scar composed of a fibrotic scar, and an astroglial scar border [6]. This scar is composed of different cell types including astrocytes, microglia, pericytes, and inflammatory and meningeal cells, together with ECM components such as fibronectin, CSPGs, and collagen among others [17–18]. To evaluate the formation of a glial scar in R- and NR-stages in response to injury we evaluated the presence of some of these markers at different days after injury.

First, we studied the expression of vimentin, an intermediate filament that is a marker of glial cells. In R-stage, vimentin was found in radial filaments located at the dorsal domain in the uninjured spinal cord (Fig. 3A, white arrowheads), as previously described (Edwards-Faret et al., 2018). Two days after injury, there was an increase in the number of cells expressing vimentin especially in the ablation gap and the regions close to the injury site (Fig. 3B, white arrowheads). Although a decrease in cells expressing vimentin was observed at 6 dpt, they are still higher than those detected in uninjured animals (Fig. 3C). For a more quantitative analysis, the region of the spinal cord surrounding the injury site was isolated and homogenized for western blot analysis. In this analysis, no change of vimentin levels was observed at 6, 10 and 20 dpt (Fig. 3D, see supplementary material, Fig. S2A). In NR-stage froglets, vimentin was only expressed in blood vessels in uninjured froglets (Fig. 3E-E’). At 10 dpt, vimentin was detected in cells with radial processes at the edges of the lesion (Fig. 3F-F’, white arrowheads) and later, at 20 dpt, many of the vimentin positive cells with radial processes were now present in the ablation gap, most probably representing glial cells forming a glial scar (Fig. 3G-G’). The observed rise in vimentin correlates with the increase in radial processes previously detected by EM analysis, in which we observed processes with intermediate filaments at the edge of the lesion (Fig. 2K-L). This increase in the levels of vimentin at later days was confirmed by western blot analysis (Fig. 3H, see supplementary material, Fig. S2B).

The presence of the ECM components typical of the glial scar in mammals was evaluated. Fibronectin is expressed in the meninges in uninjured conditions in R-stage (Fig. 3I). However, at 6 dpt a clear increase in fibronectin was detected in cells that seal the rostral and caudal stump, most probably corresponding
to meningeal cells, and was also found in more cells in the injury site (Fig. 3J). This increase is transient, and the levels of fibronectin return to almost normal at 10 dpt, being only expressed in the meningeal layer of the spinal cord (Fig. 3K). Fibronectin was almost not detected in froglets (Fig. 3L), and a similar response but more delayed, was observed. At 10 dpt, there is an increase in fibronectin deposition in the lesion site in the rostral and caudal stumps (Fig. 3M), and the levels are normal around 30 dpt (Fig. 3N). Similarly, we performed analysis of CSPGs, in NR-stages. Uninjured froglets showed expression of CSPGs only in blood vessels and vertebrae (Fig. 3O), and after injury a clear increase in the lesion site was observed, and was still present at 40 dpt (Fig. 3P-P’). CSPGs were not detected in the spinal cord before or after injury in R-stage animals (data not shown). For further analysis of collagen deposition, spinal cord sections were stained with Acid Fuchsin Orange G (AFOG), which labels collagen in blue, cells in orange and fibrin in red. Collagen was expressed in the meningeal layer in uninjured R-stage and froglets (see arrowheads, Fig. 3Q and T-T’). However, at 6 dpt the levels of collagen increased in the lesion site in R-stage (Fig. 3R), and at 10 dpt in froglets (Fig. 3U-U’). Interestingly, in R-stages the levels of collagen decreased, at 10 dpt (Fig. 3S), but high levels of collagen were still present in the lesion site in froglets at 20 dpt (Fig. 3V-V’).

For an unbiased comparison of the expression of glial markers in response to injury in R- and NR-stages we explored a data set from a high-throughput transcriptome analysis performed previously [49]. We studied the levels of expression of 8 transcripts including: the intermediate filaments vimentin (aloalleles a and b) and nestin, the ECM components versican, tenascin-C, fibronectin, collagen type 1 alpha 2, and the enzyme chondroitin 4-sulfotransferase, that is important for the synthesis of CSPGs. Of note, we found that SCI increased significantly the levels of these transcripts at 6 dpt in NR-stage froglets, but not in R-stage animals (Fig. 3W), providing further support to the different glial response in both stages.

In summary, we observed a scar formation in response to injury in NR-stage, which is absent in R-stage. A clear difference in the expression of glial scar makers in response to injury was found in R- and NR-stages. On the one hand, we found a transient increase of vimentin, fibronectin and collagen proteins in R-stages, and no important changes at the RNA levels of glia scar markers. On the contrary, in froglets, a delayed and sustained increase in the protein levels of vimentin, collagen, and CSPG was observed, together with a steady increase of their mRNAs levels.

**Characterization of the zGFAP::EGFP transgenic line**

For a better understanding of the cellular response triggered by SCI in R- and NR-stages, we decided to prepare a transgenic line that could label most of the cells in the central canal. Although it has been demonstrated that a GFAP gene was lost in *Xenopus* during anuran evolution [50], we decided to use the zebrafish GFAP (zGFAP) regulatory promoter regions to prepare a transgenic line in *Xenopus* because of the following reasons: i) based on evolutionary conservation, it is very possible that the main regulators of the gene-regulatory networks operating in glial cells are maintained, because of that we hypothesized that the regulatory promoter regions of the zebrafish GFAP gene could drive expression of a transgene in the same cells in which it is active in zebrafish; ii) a transgenic line using a 11.6 kb region of zGFAP regulatory sequences had been reported and showed proper expression of the transgene in glial and
neural progenitor cells in zebrafish [51], and iii) GFAP is usually expressed in many of the cells that are present in the CC including among others radial glial cells, neural stem, neural progenitors, astrocytes and ependymal cells [52].

Before the generation of the transgenic line, and to test our assumption that the zGFAP promoter will drive proper expression in *X. laevis* spinal cord, we electroporated the spinal cord with a construct in which EGFP expression is driven by the zGFAP regulatory sequences (zGFAP::EGFP), or a control transgene driven the expression of EGFP under a constitutive active promoter (CAG::EGFP). Electroporation of CAG::EGFP revealed an abundant and ubiquitous expression in most of the cells of the spinal cord (see supplementary material, Fig. S3A-C); compared to a more specific and selective expression after electroporation of the zGFAP::EGFP construct, which labeled a specific group of cells in the spinal cord that have a radial glial cell morphology (see supplementary material, Fig. S3D-F). This analysis suggests that the zebrafish regulatory regions drive proper expression in *Xenopus*. Because of this, we prepared a transgenic line in *X. laevis* using the same genomic region from the zGFAP described before [51]. The line obtained was named Xla.Tg(Dre.gfap:EGFP)Laar a, for short zGFAP::EGFP.

EGFP expression in the transgenic line was detected in the central nervous system (CNS) throughout development (see supplementary material, Fig. S3G-J'). At NF stage 43 and 50 the transgene was expressed in the retina, tectum, cerebellum and spinal cord, but not in the more anterior region of the CNS (Fig. 4A-C). Coronal sections of the cervical, thoracic and lumbar spinal cord showed expression of EGFP in cells, mainly in the dorsal portion of the spinal cord, that have a radial glia morphology, with their apical surface lining the central canal and a long projection to the meningeal layer (Fig. 4D and see supplementary material, Fig. S3K). Most EGFP+ cells also expressed Sox2, a marker of neural stem progenitor cells (NSPC), but not all Sox2+ cells expressed EGFP (Fig. 4E-F' and see supplementary material, Fig. S3L-M*). We noted that the region of the spinal cord with cells expressing EGFP corresponds to the same region containing cells that are actively proliferating in uninjured animals, as demonstrated before by the incorporation of thymidine analogues [47–48].

EGFP+ cells were also found in the spinal cord in NF stage 66 froglets, but have a very different shape and distribution. At this stage only a reduced group of EGFP+ cells are in contact with the CC, mainly on the most dorsal portion (Fig. 4G and I), and extend a dense array of projections towards the meningeal layer (Fig. 4G, I and I'). The most abundant cells expressing EGFP correspond to cells that are not in contact with the CC, but also have cellular projections (Fig. 4G, I, I* arrowheads). As shown previously, low levels of Sox2 expression were detected in cells lining the central canal, particularly in the subependymal layer co-localizing with EGFP (Fig. 4H,I*). Most EGFP+ cells at NF stage 66 co-expressed the Brain Lipid Binding Protein (BLBP) (Fig. 4J-L'), and glutamine synthase (GS) (Fig. 4M-O'), two markers of radial glial cells during early development, and markers of astrocytes at later stages. Base on their morphology, location, and co-expression of other markers we propose that at NF stage 66 most EGFP+ cells at the subependymal layer correspond to astrocytes.
For a more accurate identification and morphological characterization of the cells expressing EGFP in R-stage, we carried out immunogold staining using anti-EGFP antibodies. Gold particles were found in the cytoplasm of cells that are in contact with the central canal (Fig. 4P) and contain intermediate filaments (Fig. 4P',P''). Gold particles were also found on cellular projections that were in direct contact with blood vessels on the meningeal side of the spinal cord (Fig. 4Q). Based on their morphology, co-expression of Sox2 and proliferative activity we envision that most EGFP+ cells in the spinal cord of R-stage animals correspond to NSPC with a radial glial morphology. According to our previous characterization of the cells lining the central canal, EGFP+ cells in the R-stage correspond to cells type II and III and is almost not detected in froglets [48].

To unequivocally address the identity of EGFP+ cells in the zGFAP::EGFP transgenic animals at R-stage (NF stage 50) we separated EGFP+ and EGFP− cells using fluorescent activated cell sorter (FACS), and performed RNAseq in each population. Total RNA from EGFP+ and EGFP− cells was sequenced, and the reads mapped. Bioinformatics analysis (see supplementary material Fig. S4A) identified 1,718 transcripts with different levels of expression between EGFP+ and EGFP− cells, 147 of them enriched in EGFP+ cells, including EGFP with the highest fold of change, and 1,571 that showed lower levels of expression in EGFP+ cells (see supplementary material, Fig. S4B). Importantly, gene ontology analysis of the genes enriched in EGFP+ cells showed that the two main categories of biological processes enriched correspond to neural precursor cell identity and stem cell proliferation categories (Fig. 4R). Furthermore, a cluster dendrogram analysis comparing the gene profile of EGFP+ and EGFP− cells with a database of different cells types of the CNS [53] revealed a close correlation between EGFP+ cells and astrocytes, that probably include a radial glial cell signature (Fig. 4S). This molecular analysis confirmed that most of the EGFP+ cells in the zGFAP::EGFP transgenic line correspond to NSPC at NF stage 50, and validated these transgenic animals as a bona fide tool to study the response of NSPC to SCI.

In summary, the use of the zebrafish regulatory regions of GFAP allowed the generation of a X. laevis transgenic line in which EGFP expression recapitulates the expected pattern in the CNS. In R-stages, it is expressed mainly in NSPC with a radial morphology, and later in NR-stages is found primarily in astrocytes, providing a useful and reliable tool to study and characterize the function of these cells in different developmental and regenerative contexts.

**Response of neural stem progenitor cells to spinal cord injury**

We used the transgenic line described above to study the response, and function of NSPC after SCI in R-stage animals. To evaluate the proliferative response of these cells, transected and sham controls animals were incubated with a pulse of 5-Ethynyl-2´-deoxyuridine (EdU) between 20 and 36 hours after injury (Fig. 5A). Low levels of EdU incorporation in EGFP+ cells were observed in sham operated animals (Fig. 5B,C). Contrary to that, a massive proliferation of EGFP+ cells was observed after SCI (Fig. 5B,C). As a control, EdU+ cells were counted in the intestine, an organ with a high proliferation rate, and no change
on the proliferative rate was observed (see supplementary material Fig. S5A-C), indicating that the activation of proliferation raised by SCI was specific for NSPC in the spinal cord. These results are very similar to those reported for the activation of Sox2+ cells in R-stage [47] giving further support to the finding that most EGFP+ cells at this stage co-express Sox2.

To study in detail the response of EGFP+ cells, we fixed animals at different days, and performed immunofluorescence against EGFP in longitudinal sections. To allow a more detailed analysis of the cellular responses in the ablation gap, we performed a resection of the spinal cord that implicates the complete removal of a piece of the spinal cord of approximately 200 µm. In uninjured animals, but now in a longitudinal section, the EGFP+ cells showed their radial morphology and its dorsal and lateral location (Fig. 5D-5D*). At 2 days post resection (dpr), both ends of the spinal cord were approximately 200 µm apart (Fig. 5E), and many round shaped EGFP+ cells were found at the edges of the rostral and caudal stumps (Fig. 5E',E*, arrowheads). Interestingly, at 6 dpr, EGFP+ cellular processes started to extend from the rostral and caudal stumps towards the ablation gap (Fig. 5F-F*, arrowheads). At 7 dpr some of these processes were even able to cross the complete ablation gap (Fig. 5G,G', arrowheads). At 10 dpr, EGFP+ cells populated the injured site (Fig. 5H,H') and some reorganization of the central canal is observed (Fig. 5H'). At 20 dpr, some recovery of the anatomy of the spinal cord was observed (Fig. 1T, and [46–47]). EGFP+ cells were starting to acquire their normal location; however, a radial glial morphology was not observed, and these cells were now present in the ventral level (Fig. 5I-I*).

Regarding the cellular processes extending into the ablation gap observed at 6–7 dpr we hypothesized two alternatives: i) they could correspond to glial extensions that can provide a substrate for axon growth and pathfinding, something that has been proposed before [42] or ii) in agreement with our previous findings on the role of neurogenesis on spinal cord regeneration [47], these processes could be axons from the new neurons generated from the EGFP+ cells, that because of the half-life of the EGFP protein allowed the study of the cell fate of the EGFP+ cells. To analyze these two possibilities, we performed immunofluorescence against acetylated tubulin, which labels axons and cilia, in the same time points depicted above. As expected, in uninjured animals, acetylated tubulin does not co-localized with EGFP in axons (Fig. 5J-J*, see arrowheads), but there is co-localization in cells in the central canal probably because acetylated tubulin is present in ciliated cells (Fig. 5J-J*, see arrows). However, at two days after injury a clear co-localization of acetylated tubulin with EGFP was detected, primarily at the edge of the stumps in structures that are reminiscent of axons and axon growth cones (Fig. 5K-K*, arrowheads). Importantly, at 6 and 7 dpr most of the EGFP+ cellular processes extending into the ablation gap co-localized with acetylated tubulin (Fig. 5L-L*,M-M*). Something similar was observed at 10 dpr (Fig. 5N-N*). An expression pattern of acetylated tubulin like the uninjured spinal cord is observed at 20 dpr, however, some co-localization of acetylated tubulin and EGFP was still observed in the axonal tracts (Fig. 5O-O*, see arrowheads).

These results support the hypothesis that the EGFP+ cellular processes crossing the ablation gap correspond to axons because of their morphology, and the co-expression of acetylated tubulin. The fact
that they are EGFP+ indicates that most probably they correspond to new neurons formed from the NSPC present in the central canal.

**Fate and function of neural stem progenitor cells in spinal cord regeneration**

To further evaluate the fate of NSPC, we took advantage of the persistence of EGFP expression. EGFP+ and EGFP− cells were isolated by FACS before and after injury, and the expression levels of the following markers were measured by RT-qPCR (Fig. 6A): i) sox2 and nestin, for NSPC; ii) neurogenin3, *achaete-scute homolog 1* (Ascl1), neurogenin2a, doublecortin (Dcx), and neuroD1, for neuronal precursors and neurogenic differentiation; (iii) aldehyde dehydrogenase1l1 (aldh1l1) and vimentin-a, for astrocytes; and the myelin binding protein (Mbp), and Sox10 for oligodendrocytes.

An increase of approximately 450 and 130 times, was observed in the ratio of EGFP levels between EGFP+ and EGFP− cells, at 2 and 6 dpt, respectively. These ratios were many times higher than the ratios detected in uninjured animals (Fig. 6B), probably explained by the increase on the total number of EGFP+/NSPC, because of its massive proliferation induced by SCI (Fig. 5C, and [47]). Supporting the increase in the proliferation of NSPC induced by transection, we observed higher ratios of sox2 and nestin, between EGFP+ and EGFP− cells, at 2 and 6 dpt (Fig. 6C,D). The most probable explanation for this rise is an increase in the number of EGFP+ cells; however, we cannot discard the possibility that the higher ratios were also explained by an increased expression of these genes in each EGFP+ cell.

Interestingly, the early neurogenic markers Ascl1, Neurogenin2a, Neurogenin3, NeuroD1 and Dcx were also increased at 2 dpt, and in some cases also at 6 dpt (Fig. 6E-I). A similar response was observed in astrocytes marker such as vimentin-a and Aldh1l1 (Fig. 6J,K). As an indication that the transgene zGFAP::EGFP is not expressed in oligodendrocytes, lower levels of Sox10 and Mbp were detected in EGFP+ than EGFP− cells, in uninjured animals, and these levels were even smaller at 2 dpt, probably as a consequence of the enrichment in the neuronal, and astrocytic lineage (Fig. 6L,M). In line with the analysis depicted above, these results showed that SCI activates NSPC proliferation, followed by the fate of this neural precursor to the neurogenic and/or astrocytic lineage, but not to oligodendrocytes.

To study the function of NSPC we prepare a transgenic line with the nitroreductase/metronidazol (NTR/MTZ) system in order to specifically ablate these cells [54]. Spinal cord electroporation with a zGFAP::mCherry-NTR construct (see supplementary material, Fig. S6A) followed by incubation with 10 mM MTZ or vehicle (see supplementary material, Fig. S6B) showed a very effective ablation of mCherry+ cells once animals were treated with MTZ compared with vehicle treatment (see supplementary material, Fig. S6C-R). Based on these results we prepared the transgenic line Xla.Tg(Dre.gfap:mCherry-Nitroreductase)Larra (see supplementary material, Fig.S5S-U), for short zGFAP::mCherry-NTR.

We use this line to evaluate the effects of NSPC ablation in the ability of R-stage animals to regenerate the spinal cord and recover their swimming ability. For this, four groups of animals were considered: i)
sham operated animals treated with vehicle or MTZ, and ii) resected animals treated with vehicle or MTZ. Animals were incubated with vehicle or MTZ for 1 week before sham operation or spinal cord transection (Fig. 7A). Efficient ablation of mCherry\(^+\) cells was attained at 7 days after incubation with MTZ (Fig. 7B-G). After transection, we measured the swimming ability of each group. Treatment with MTZ has no effect on the ability of sham-operated animals to maintain their swimming ability compared to vehicle treated animals (Fig. 7H, and data not shown). Importantly, at 15 and 25 dpr animals incubated with MTZ showed a diminished swimming ability compared to vehicle treated siblings (Fig. 7H, compare red and green boxes). In addition, we performed Sox2 immunostaining in sections of the spinal cord at 30 dpt, and found that treatment with MTZ, as expected, resulted in a strong reduction of Sox2\(^+\) cells precluding the proper regeneration of the spinal (Fig. 7I-L). These results indicate that NSPC cells are necessary for regeneration of the spinal cord of NF stage 50.

**Discussion**

We compared the cellular response to spinal cord injury in R- and NR-stage of *Xenopus laevis*, particularly looking at the response on the cells lining the spinal cord central canal. Previously, we have described that most cells lining the central canal express Sox2 \[46\], are activated to proliferate in response to injury, and are required for spinal cord regeneration in R-stages but not in NR-stages \[47\]. In addition, we found that progressive changes in the cell types lining the central canal of the spinal cord during metamorphosis correlate with the regenerative capacities \[48\]. Here, using electron microscopy and immunofluorescence analysis we found several differences in the cellular response to spinal cord injury between R- and NR-stage. For a comprehensive role of NSPC and glial cells in the regenerative abilities of *Xenopus*, we generated a reporter line with the regulatory elements of the zebrafish GFAP. Interestingly, in R-stage the transgene is expresses in NSPC, however after metamorphosis, zGFAP\(^+\) cells resemble a differentiated population of astrocytes with radial morphology. Further analysis of the zGFAP\(^+\) cells in R-stages, demonstrated that those cells are NSPC, make new neurons after injury, and are necessary for the functional regeneration of the spinal cord in R-stage.

**Cellular response in R- and NR-stages**

Of note, the cells lining the central canal at 2 dpt in R-stages, looks very healthy and normal. In contrast, central canal cells in NR-stages suffer an important loss of internal organelles, and therefore the main structure of the central canal was heavily affected at early days after injury. Instead, abundant red blood cells, which were almost absent in R-stages, filled the injury site. One of the first regenerative steps in R-stages is the rapid close of the rostral and caudal stumps by the cells lining the central canal. This fast response is important to maintain a structural homeostasis of the injured spinal cord at R-stage. Interestingly, it resembles the first step during wound healing, where a platelet plug is initially formed to maintain hemostasis \[55\]. In correlation with our previous studies \[48\], the ependymal cells closing the rostral stump were characterized by a high nucleus/cytoplasm ratio and lax chromatin. In addition, those cells have lost their apical/basal disposition, due to the fact that neither the cilia, nor the desmosome
type junctions are observed. Although the lack of a cilium in those ependymal cells, centriolar satellites structures were observed, such structures has been described as essential granules around centrosomes which are involved in ciliogenesis [56], meaning that the cilia in those ependymal cells is under construction. We previously described different cell types lining the regenerative CC (Type I, II, III, IV and V). After injury we identified few of them (Type III), however, most of the cell types were not clearly identified after injury, probably because the activation state of these cells did not allow us to differentiate between the different CC cell types in R-stage.

An active process of cellular extrusion was observed at 2 days after injury in R-stage, cells lining the central canal were extruded into the lumen of the central canal. Interestingly, extrusion was mainly detected between 2 and 6 days after the injury. Cell extrusion can act as a regulator from epithelial homeostasis by removing apoptotic cells [57], altering cellular position to regulate development, or to determine cell fate, as happens with neuroblast delamination before initiating the neurogenic divisions in *Drosophila* [58]. Many of the cells extruded into the central canal have a morphology similar to the cells lining the central canal, none of these processes was observed in NR-stages. In addition, it is also possible to envision that the cells extruded into the central canal can then migrate to the injury site, and collaborate on spinal cord reconstruction.

The infiltration by immune cells was very scarce in R-stages, besides few red blood cells, and few macrophages at the rostral and caudal stumps at 2 dpt, no further immune cells were detected in R-stages. Instead, in NR-stages, cell infiltration constitutes one of the first processes, resulting in an injured site full with red blood cells at 2 dpt, followed by a peak of macrophages phagocyting the red blood cells at the injured site at 6 dpt, similar to mice and rats evidence [59]. However, at difference with the mammalian counterpart in which macrophages can be found even after 42 days post injury [59], in froglets the macrophages response is limited in time, and after 10 and 20 dpt no more macrophages were found in the injured spinal cords.

Another hallmark of the response in the R-stage is the formation of rosettes structures composed by cells similar to the ones lining the central canal. This organization mimics a neural tube like structure; cells have adherent junctions, unlike the desmosomes observed in ependymal cells under basal conditions [48], and are surrounded by a basal lamina. These structures are no longer observed with the progress of regeneration, most probably they collapse in the formation of the new central canal. In addition, at 10 days after injury in R-stages, many cells with neuronal characteristics including abundant unmyelinated axons, synaptic vesicles and postsynaptic densities were founded inside the lumen of the central canal. In contrast, in NR-stages the response at 6–10 dpt was characterized by a stabilization and reconstruction of the epithelia lining the central canal at the rostral and caudal stumps, and the injured site was still populated by macrophages. In addition, glial processes were already located surrounding the stumps and not crossing the injured site in NR-stages.

Three to four weeks after injury, a complete, although not perfect, regeneration of the spinal cord was observed in R-stage, with a proper reconnection of the central canal, and the restructuring of the CC. A
clean central canal with just few extruded cells correlates with the full recovery of the swimming capabilities in R-stage [47]. In contrast, in the NR-stage, axons crossing the injured site are completely absent, and the continuity of the central canal is not restored. At this stage, the injured site is mainly populated by fibroblast-like cells, and filled with ECM components, including collagen and CSPGs. This is confirmed by the increase on vimentin, and other glial scar components.

**Role of NSPC in spinal cord regeneration**

We previously described the cells lining the central canal of the spinal cord as possible neural progenitor cells due to the expression of the transcription factor Sox2/3 and their requirement to the regenerative process in R-stages [46–47]. Interestingly, the TEM analysis reveals a multi-faceted role of central canal cells, not only proliferating, in addition being extruding into the central canal, forming rosettes to reconnect the central canal. To understand the identity of those cells, we generated the transgenic line based on the regulatory elements of the GFAP from zebrafish (zGFAP). GFAP has been robustly present in radial glial cells during embryonic development, and specific astrocytes subpopulations in adult mammals. Radial glial cells are the primary NSPCs during mammalian CNS development [52]. In addition, GFAP is expressed in radial glial cells in non-mammalian models such as zebrafish [51] and axolotl [60]. Although it has been reported the loss of the gfap gene in *Xenopus laevis* during anuran evolution [50], we decided to use the regulatory elements of the GFAP from zebrafish [51]. Interestingly, zGFAP drives expression in *Xenopus laevis* CNS, in addition, cells expressing EGFP under the control of the zGFAP have radial glial features regarding their: i) morphology, with an apical process contacting the central canal, and extending a long radial process to contact blood vessels, and the meningeal layer; ii) location, surrounding the central canal in the brain regions such optic tectum, hindbrain and in the spinal cord; iii) timing of expression, EGFP is expressed at early neurulation stages of brain and spinal cord development; iv) molecular makers, EGFP* cells express neural precursor proliferation markers, such as Sox2/3 and Nestin, and express the molecular signature of NSPC, and v) cells expressing EGFP contain intermediate filament, as revealed by the immungold staining. This suggests that although the gfap gene is absent in frogs, the regulatory regions of the zGFAP gene are activated specifically in NSPC with a radial glial cells morphology.

Moreover, according to our previous characterization of the cells lining the central canal in the spinal cord of R-and NR-stages, the EGFP* cells correspond to cell types II (dorsal) and III (lateral) in R-stages, and therefore the highest proliferative cells and the proposed source of cells for repair in case of damage [48]. In NR-stages, the population of EGFP* cells lining the central canal were located only at the dorsal side of the spinal cord, consequently, corresponding to the dorsal radial glial-like cells (Edwards-Faret et al., 2018). However, most of the EGFP* cells at this stage were found in a subependymal layer and express the radial glial marker BLBP but also the astrocyte marker GS [61], demonstrating that one of the main source of proliferative cells is considerable reduced in NR-stages, and instead zGFAP::EGFP* cells are a more differentiated type of astroglial cells.
Taking the advantage that the zGFAP::EGFP transgene allows the labeling of the NSPC, we studied in more detail how they respond to injury. First, as described previously, they responded by a massive proliferation [46–47]. In addition, zGFAP::EGFP+ cells extended long EGFP+ processes into the injured site, those processes co-localized with the neuronal marker acetylated tubulin, indicating that these NSPC differentiate into neurons, confirming the role of neurogenesis in the regenerative process. In addition, we follow the fate of these cells after injury, and found a neurogenic response. After injury, we detected an increase in cells expressing neurogenic factors, or an increase on the expression of these genes in each cell. In particular, we found an increase in Ascl1, Neurogenin 2a, Neurogenin 3 and NeuroD [62], at 2 and 6 dpt. Concomitant with that, we also found an increase in astrocytic markers (vimentin and Aldh1l1) that could be explained because of the self-renovation of radial glial cells, or because some NSPC are fated to the astrocytic lineage. On the contrary, no increase of oligodendrocyte markers was detected. This evidence confirms that EGFP+ cells act as NSPC and they are fated into neurons and astrocytes. Mammals also have a small population of NSPC residing in the ependymal layer lining the central canal of the spinal cord in mice, however, after injury those cells are activated and mostly differentiate into glial cells and oligodendrocytes [10]. The finding that zGFAP::EGFP positive cells are activated to make new neurons and astrocytes, does not preclude the possibility that they can also contribute by providing a permissive substrate or “bridge” for axon regeneration as described as bridge in zebrafish [36–37].

Furthermore, selective cell ablation of the zGFAP+ cells in the CNS in R-stage revealed that after spinal cord injury animals are not able to recover their swim ability. Cell ablation using the Nitroreductase/Metronidazole system has been previously proved to induce specific and selective cell ablation [54] including in a retinal degeneration model in Xenopus laevis [63]. This finding confirm that zGFAP+ NSPC cells are necessary for the functional regeneration of the spinal cord in R-stages of Xenopus laevis.

Conclusions

Cellular response to spinal cord injury dramatically differs between R-stage and NR-stages of Xenopus laevis. The regenerative process proceed by the activation of cells lining the central canal, here we described that cell types II and III [48] are NSPC. Their role in the anatomical recovery of the central canal and spinal cord reconnection is correlated with their requirement for the functional recovery of the R-stage swim abilities. On the contrary, this ability is lost in NR-stage that respond with the formation of a glial scar and zGFAP+ cells are radial astrocytes. One future challenge is to study zGFAP+ cells in NR-stage, and evaluate the possibility to re-activate zGFAP+ cells in this stage to promote spinal cord regeneration. Lastly, there is still unclear how the regulatory elements from the zebrafish GFAP respond in the absence of gfap gene in radial glial cells in Xenopus laevis.

Material And Methods

Animals
R-stages (NF stage 50) and NR-stage (stage 66) of *Xenopus laevis* were produced by natural mating of wild-type mature male and female frogs obtained from Nasco. Animals husbandry was performed as previously described [40]. All animal procedures were approved by the Committee on Bioethics and Biosafety from the Faculty of Biological Sciences, Pontificia Universidad Católica de Chile (Protocol CBB-004/2013), according to Chilean’s Protection of Animals Act 20380 (2009) and the Guide for the Care and Use of Laboratory Animals (National Research Council, Eighth Edition, 2011).

**Constructs**

The CAG-GFP plasmid was obtained from Addgene (Addgene plasmid # 16664). The pEGFP-gfap (Intron1/5’/Exon1-zebrafish) plasmid was a donation from Dr. Pamela Raymond (Addgene, plasmid # 39761), and to facilitate its name was shortened to zGFAP::EGFP. The Osx::mCherry-Nitroreductase plasmid was a donation from Dr. Kenneth Poss (Duke University, USA). The zGFAP::mCherry-Nitroreductase was subcloned by the company Genewiz, by taking the regulatory elements from the zebrafish GFAP [pEGFP-gfap (Intron1/5’/Exon1-zebrafish) and cloned into the Osx::mCherry-Nitroreductase, by replacing the Osx promoter.

**Transgenesis**

The zGFAP::EGFP transgenic line was generated from zGFAP::EGFP plasmid. Transgenesis was adapted from [64]. Briefly, eggs were obtained by in vitro fertilization from adult wild type Xenopus laevis females. Dejellied one-cell stage embryos were injected with a mixture of linearized plasmid pEGFP-gfap (Intron1/5’/Exon1-zebrafish) or zGFAP::mCherry-Nitroreductase (generated by mixed with sperm nuclei and eggs extract). Embryos were incubated at 18 °C. At NF stage 35 transgenic embryos expressing EGFP or mCherry were selected under a Nikon SMZ-1500 stereoscope. Once embryos reached stage 42, tadpoles were raised at 20–21 °C for 10–12 months until sexual maturation.

**EdU injection and Click-iT staining**

To identify proliferating cells in the zGFAP::EGFP+ cells, R-stage animals (n = 3, each stage) received one intracoelomic (i.c.) injection of 50 mg of EdU per gram of body weight at 16 hr after 2 dpt or sham control surgery. Animals were anesthetized and fixed by immersion in 4% paraformaldehyde (PFA) as previously described [40]. To analyze EdU labeled cells in zGFAP::EGFP+ R-stage, skin and dorsal muscle were dissected for whole mount spinal cord preparation. EdU labeling was performed using the Click-iT EdU Alexa Fluor 555 Imaging Kit (Thermo Fisher Scientific, cat. no. C10338) according to manufacturer’s technical information. Briefly, whole mount spinal cords were permeabilized in PBST 0.5% and then incubated in blocking solution for 30 min at room temperature following by an incubation in the Click-iT EdU reaction cocktail for 1 hr. Click it was followed by Immunofluorescence against EGFP and DNA staining with Hoechst (1:10,000). Whole mount spinal cords were mounting with vectashield (Vector Laboratories, H-1000). Confocal z-stack images were taken on an Olympus (Fluoview FV10i) microscope and images were analyzed with Adobe Photoshop (Adobe Systems, San Jose, CA).

**Transmission electron microscopy**
For transmission electron microscopy (TEM) analysis, uninjured (ut) R-stage and NR-stage animals and after 2, 6, 10 and 20 after spinal cord injury (n = 2/3 each point) were anesthetized and immersed (R-stage) or perfused with 0.83 PBS (NR-stage) followed by incubation in 2% PFA and 2.5% glutaraldehyde (EMS, Hatfield, PA). Spinal cords were micro-dissected and post-fixed overnight in the same fixative. Spinal cords were processes as described before [48]. Briefly, spinal cords at different days post injury were post-fixed in 2% osmium for 2 hr, rinsed, dehydrated, and embedded in araldite (Durcupan; Fluka, Buchs, Switzerland). Semi-thin horizontal sections (1.5 mm) were cut with a diamond knife and stained with toluidine blue. To study the cellular response to injury at the different days and stages, ultrathin sections (60–70 nm) were cut with a diamond knife, stained with lead citrate, and examined under a transmission electron microscope (TEM) (Tecnai Spirit G2; FEI, Eindhoven, The Netherlands) by using a digital camera (Morada, Soft Imaging System; Olympus, Tokyo, Japan). Brightness and contrast adjustment of the pictures was performed with Adobe Photoshop (Adobe Systems, San Jose, CA).

**Immunogold staining**

For pre-embedding staining, zGFAP::EGFP+ R-stage animals (n = 3) were anesthetized and immersed 4% PFA and 0.5% glutaraldehyde. EGFP immunostaining on semithin sections was performed as described [48]. Pre-embedding immunogold staining was performed by incubating semithin sections in primary antibody (1:200 for anti-EGFP) followed by appropriate colloidal gold-conjugated secondary antibodies (1:50; UltraSmall; Aurion, Wageningen, The Netherlands).

**Spinal cord injury**

We used two methods to induce spinal cord injury, spinal cord transection or spinal cord resection as described before [40]. Briefly, for spinal cord transection in R-stages, animals were anesthetized, then the skin and dorsal muscles were opened at the mid-thoracic level, and the spinal cord was fully transected with a clean cut at the thoracic level. In NR-stage, after anesthesia, a small incision was made at the skin and dorsal muscle, followed by laminectomy of the dorsal portion of the sixth vertebra, a complete transection through the spinal cord was performed using microdissection scissors. For spinal cord resection, two incisions were made at the spinal cord and a whole section (150–200 µm) was removed in R-stage and NR-stage. Control surgery (sham) were performed at R-stage and NR-stage by an incision at the dorsal skin and muscles but without injured the spinal cord. After surgery, animals were transferred into their tanks with 0.1 x Barth supplemented with antibiotics (penicillin and streptomycin).

**Spinal cord electroporation**

R-stage animals were electroporated in the spinal cord as desced before [40]. Briefly, animals were anesthetized in 0.02% MS222, and the plasmid pCAG:EGFP at 2.5 µg/µl (Addgene plasmid # 16664) or zGFAP::EGFP construct at 2.5 µg/µl (Addgene plasmid # 39761) was injected with a glass capillary into the central canal of the spinal cord. Voltage pulses were applied with a Grass SD9 stimulator (GrassTele-factor, USA) across the back using homemade platinum electrodes (5 pulses of 35V in each polarity, 50 ms pulse length and 200 pps frequency). Animals were transferred into 0.1x Barth containing antibiotics. Screening of EGFP was perform 24 hr after electroporation.
**Immunofluorescence**

R-stage animals were anesthetized and fixed by immersion in 4% PFA and NR-stage were perfused with 0.83 PBS followed by 4% PFA, the NR-stage spinal cord were post-fixed in the same fixative during overnight. Immunofluorescence was performed as described before [48]. Briefly, spinal cords were embedded in increasing sucrose solutions (5–10–20%), followed by optimal cutting temperature compound (OCT, Tissue Tek®), and frozen in liquid nitrogen. Transversal or sagittal cryosectioned at 10 mm were prepared. Sections were permeabilized in phosphate-buffered saline (PBS; pH 7.4) containing 0.2% Triton X-100 (PBST), then incubated in blocking solution (PBST with 10% goat serum) (blocking solution) for 30 min at room temperature. Sections were incubated with the corresponding primary antibody diluted in blocking solution overnight. Primary antibodies were acetylated tubulin (1:200; T7451-Sigma); BLBP (1:200, ABN14-EMD Millipore); GS (1:200, MAB302-EMD Millipore); CSPGs (1:100, C8035-Sigma); GFP (1:200, ab6556-Abcam); Fibronectin (1:200, F3648-Sigma); Sox2 (1:200, 4900S-Cell Signaling Technology); Vimentin (1:50, 14h7-Developmental Studies Hybridoma Bank). Samples were incubated with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes, Eugene, OR) at 1:500 in the blocking solution for 2 hr at room temperature. Immunofluorescence were followed by DNA staining with Hoechst (1:10.000) and mounting with vectashield (Vector Laboratories, H-1000). Samples were imaged using an Olympus (Fluoview FV10i) confocal microscope and images were analyzed with Adobe Photoshop.

**Acid Fuchsin with Orange-G staining**

Collagen analysis was performed in 10 µm horizontal paraffin sections of R-stage (NF stage 50) and NR-stage (NF stage 66) uninjured spinal cord after 6 and 10 days post transection (dpt) in R-stage and after 10 and 20 dpt in NR-stage of three different replicates at each stage and day. For AFOG (Acid Fuchsin with Orange-G) staining, slides were deparaffinized in xylol, followed by rehydration in decreasing ethanol solutions (100% at 40%) and washed in distilled water. Overnight incubation in Bouin's solution, followed by 30 min wash in running water. Then the slides were incubated in 1% phosphomolybdic acid (10%), wash in running distilled water and staining with AFOG (aniline blue, orange G and acid fuchsin, in a ratio 1: 2: 3 respectively), and incubation in 0.5% glacial acetic acid. Sections were dehydrated with 96% and 100% ethanol, and xylol, slides were mounted with entellan mounting medium and covered with a coverslip. The slides were dry for 1 day at room temperature.

**Western Blot**

For western blotting spinal cords from R-stage 50 (n = 12) and NR-stage were isolated in uninjured animals (ui) and after 2, 6, 20 and 20 days post injury (dpt). Isolated spinal cords were homogenized in RIPA lysis buffer with protease inhibitors (benzamidine 1 µM; leupeptin 5 µg/ml; Na₃VO₄ 200 µM; phenylmethylsulfonyl fluoride 200 µM and sodium pyrophosphate 200 µM). Western blot was performed as described previously [46–47]. Proteins were quantified with the protein assay kit (Thermo Scientific) and 20 µg of protein was loaded in each lane. Primary antibodies against vimentin (1:500, 14h7-Developmental Studies Hybridoma Bank) and GAPDH (1:10000, EMD-Millipore) were used. Densitometry
analysis of Vimentin and GAPDH bands was performed with ImageJ (National Institutes of Health, Bethesda, MD, USA) and in R-stage and NR-stage the Vimentin/GAPDH ratio was normalized to the uninjured (ui) control.

**Cell dissociation and FACS**

Spinal cords from EGFP\(^+\) and EGFP\(^-\) animals at R-stage from the transgenic line zGFAP::EGFP were dissected from anesthetized animals in MS-222 (n = 60), and an enzymatic dissociation in StemPro Accutase (Gibco) in a soft shaking (1–2 speed in a vortex) at room temperature for 30 min. The cell suspension was centrifuged and the cells were resuspended in dissociation buffer (100 µg/mL DNAse I, 5 mM MgCl\(_2\), 1X HBSS) and the samples keep in the tubes on ice until Fluorescence-activated cell sorting (FACS). For FACS, cells were identified based on size, granularity and EGFP expression. We obtained a 90,1% of viability, based on propidium iodide negative staining. From this cell population, 51% were EGFP + cells and 33% were EGFP- cells and 6,1% of cells between EGFP + and EGFP- cells was discarded from the analysis in order to improve the purification of both cell samples.

**RNA extraction**

For the purification of total RNA from EGFP\(^+\) and EGFP- cells, the commercial kit (RNeasy Mini Kit) was used according to the manufacturer's. Total RNA was isolated and eluted in water. A treatment with DNase I (QIAGEN) was included. RNA concentration was measured using Nanodrop (Thermo Scientific).

**RNAseq**

After cell populations EGFP + and EGFP- were separated using fluorescent activated cell sorter (FACS), total RNA extractions were performed and PolyA + RNAseq libraries were prepared. Later, both libraries were sequenced using Illumina Hiseq4000 platform and obtained a mean of 34,8 (EGFP\(^-\)) and 38,6 (EGFP\(^+\)) million paired-end reads. Sequence quality analysis was performed using FASTQC determining a mean quality score of 27 for reads. Posteriorly, reads were aligned to the *Xenopus laevis* J-strain 9.1 transcriptome (XL_9.1_v1.8.3.2, Xenbase) using Bowtie-RSEM with default parameters [65–66]. We reached a mapping rate of 70%, comparable between EGFP + and EGFP- cells supporting the robustness of both sequencing results. Differential gene expression among both cell populations was analyzed using DESeq [67] and following the protocol described in its manual for an experiment without replicates, we considered as differentially expressed those genes with fold change ≥ 2 or ≤ 0,5 and p-value ≤ 0,05. Gene Ontology (GO) enrichment analysis was performed using clusterProfiler [68] and testing for biological process enriched, we considered all GO terms with adjusted p-value ≤ 0,05. ClusterProfiler was also used for to visualize GO terms enriched in fourth GO level. For to identify EGFP + and EGFP- cells, we compared its expression profiles with a mouse database of different cells types (neurons, astrocytes, oligodendrocyte precursor cells, newly formed oligodendrocytes, myelinating oligodendrocytes, microglia, endothelial cells, and pericytes from mouse cerebral cortex) of the CNS (Ben Barres database [53]). Because this database correspond to a diploid animal model, for to compare we summed counts for homologous gene pairs (L and S genes) and determined differential expression for each pair using DESeq. Posteriorly, we generated a cross database join between homologous gene pairs differentially
expressed and genes with fold change ≥ 4 between cell populations of Barres Database, we choose a high fold change for to highlight differences in expression profiles among cell types. Finally, we evaluated Euclidean distance over FPKM values for to identify the more related cell population to EGFP+ cells.

**Real time qPCR**

The cDNA from two or three independent biological replicates of EGFP+ and EGFP- cells from the transgenic line zGFAP::EGFP at R-stage were prepared from uninjured animals and after 2 and 6 days post transection were synthesized using the M-MLV reverse transcriptase (Promega), and RT-qPCR was performed using Power SYBR Green (Applied Biosystems) by performing three technical replicates on two or three independent biological replicates. The relative expression ratio was calculated as described using eef1a1 (GenBank: BC043843) as a reference gene. The primers used were EGFP, Sox2, Nestin, Ascl1, Neurog2a, Neurog3, NeuroD1, Dcx, Vim-a, Aldh1I1, Sox10, Mbp. For the primers sequence please see supplementary table 1.

**Cell ablation**

For the ablation of the zGFAP: mCherry-Nitroreductase+ cells, transgenic animals at R-stage (NF stage 50) were incubated in 10 mM metronidazole (MTZ) prepared in chlorine-free water for 7 days, prior to transection of the spinal cord, and kept protected from light at 21 °C. The medium was changed daily. Control animals were kept in chlorine-free water with and without metronidazole. Cell ablation was evaluated by analysis of mCherry red fluorescence in the eye under fluorescent microscope and spinal cord by confocal imaging.

**Swimming recording**

Animals swim at 1, 10, 15 and 25 days post resection or sham control surgery was tracked and recorded as described before [40, 47]. Briefly, an R-stage animal was placed in a 15-cm-diameter Petri dish filled with 100 ml of 0.1x Barth solution. After 5 min of adaptation, a video tracking started for 5 min using the ANY-maze software (Stoelting Co,Wood Dale, IL). The software recorded the trajectory and measured the swimming distance traveled by each animal and the total swimming distance was plotted against the days after injury. Once the test was completed, animal was transferred into their respective tank.

**Cell counting**

To analyze the EdU and EGFP+ cells from zGFAP::EGFP transgenic animals at R-stage after 2 dpt and 2 dps (n = 3 each group). Whole mount spinal cord were imaged under confocal microscope. Z-Stack were analyzed using a cylinder template and cells were counted in the spinal cord and intestine. Double stained cells EdU+ and EGFP+ cells ere normalized according to the corresponding area in each replicate, the average of the three replicates is shown in each graph. Statistical analyses were performed with ANOVA followed by Tukey's post hoc test, with results considered significant at p < .05.

**Statistics**
Statistical analyzes were performed with the GraphPad Prism 5 program. For the analysis of swimming capacity, the one-way ANOVA and Bonferroni post-test were used. For the analysis of zGFAP::EGFP+ cell proliferation, t-Test was performed.

**Abbreviations**

**AFOG**
Acid Fuchsin Orange G; aldh111:aldehyde dehydrogenase111; Ascl1:achaete-scute homolog 1; BLBP:Brain Lipid Binding Protein; CC:central canal; CNS:central nervous system; CSPGs:chondroitin sulfate proteoglycans; Dcx:doublecortin; dpe:days post electroporation; dpr:days post resection; dpt:days post transection; dtt:days post treatment; ECM:extracellular matrix; EdU:5-Ethynyl-2′-deoxyuridine; EGFP: Enhanced green fluorescent protein; EM:Electron Microscopy; ERG:ependymo-radial glia; FACS: Fluorescence-activated cell sorting; FPKM:fragments per kilobase of exon model per million reads mapped; GFAP:Glial fibrillary acidic protein; GO:Gene Ontology; GS:glutamine synthase; Mbp:myelin binding protein; mRNAs:Messenger RNA; MTZ:metronidazole; neurog2a:neurogenin2a; neurog3:neurogenin3; NF stage:Nieuwkoop and Faber stage; NR-stages:non-regenerative stages; NSPC:Neural Stem Progenitor Cells; NTR/MTZ:nitroreductase/metronidazole; PBS:Phosphate-buffered saline; PBST:phosphate-buffered saline with Triton X-100; PFA:paraformaldehyde; RNAseq:RNA-sequencing; Rs:resection injury; R-stage:regenerative stages; SC:spinal cord; SCI:spinal cord injury; Sh:sham operation; Sox2:SRY (sex determining region Y)-box 2; TEM:Transmission electron microscopy; ui:uninjured; vim-a:vimentin-a; *X. laevis*: Xenopus laevis; zGFAP:zebrafish GFAP; zGFAP:EGFP:construct of zebrafish GFAP regulatory elements driving EGFP and the transgenic line generated in Xenopus laevis Xla.Tg(Dre.gfap:EGFP) *Larra*

**Declarations**

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**Authors’ contributions**

**GEF:** Conceptualization, Investigation, Formal analysis, Validation, Writing - Original Draft, Writing - Review & Editing, Funding acquisition. **KGP:** Investigation. **ACS:** Investigation, Review & Editing. **JP:** Investigation, Review & Editing. **JMGV:** Investigation, Formal analysis, Review & Editing. **JL.** Supervision, Writing - Original Draft, Writing - Review & Editing, Funding acquisition. The author(s) read and approved the final manuscript.

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**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and material**

All datasets, Xenopus lines, plasmids, and other reagents are available upon request from the corresponding author.

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Figures
Figure 1

Cellular response to injury in Regenerative Stage. (A) Cartoon of spinal cord injury in NF stage 50. (B,C) Semithin sections of the (B) rostral and (C) caudal stumps at 2 days post transection (dpt) stained with methylene blue. Arrowheads in panel C showed macrophages. (D-I; K-M; O-S; U-V) Correspond to ultrathin sections observed by transmission electron microscopy. (D-I) Different regions of the spinal cord at 2 dpt; (D) cells lining the central canal (cc) closing the rostral stump (black arrowhead); (E) mitochondrial
swelling (black arrow) observed in cells from panel D; black arrowheads depict the separation between two cells; (F,G) mitotic clusters of cells (yellow shadow); (H) cell undergoing extrusion (purple shadow); (I) macrophage from panel C from the injured site. (J) Semithin section at 6 dpt. (K-M) Different regions of the spinal cord at 6 dpt; (K) cells in the central canal of the caudal stump (green shadow), without contact with ependymal cells (black arrowheads); (L) synaptic density (black arrowheads) and synaptic vesicles (white arrowheads); (M) cells forming a rosette structure in the ablation gap (red shadow). (N) Semithin section at 10 dpt. (O-S) Different regions of the spinal cord at 10 dpt; (O) a bundle of unmyelinated axons surrounded by ependymal cells (black arrowheads); (P) unmyelinated axon (orange shadow), and synaptic vesicles (white arrowheads); (Q) desmosome junction (black arrowhead) between ependymal cells next to unmyelinated axons; (R) a myelinated axon (white arrowhead) in the cc of the caudal stump; (S) neuronal nuclei in the cc of the caudal stump (black arrowhead). (T) Semithin section at 20 dpt. (U-W) Different regions of the spinal cord at 20 dpt; (U) cells in the cc (red line); (V) ependymal cells with regular shape in the regenerated spinal cord with apical mitochondria (m); (W) desmosome junctions between the regenerated ependymal cells (black arrowheads). The red dotted lines indicate the injured site (A, B, C, J, N, T).
Figure 2

Cellular response to injury in Non-Regenerative Stage. (A) Cartoon depicting the process of spinal cord injury in NF stage 66. (B) Semithin section at 2 dpt. (C-D; F-G; I-L; N-P) Correspond to ultrathin sections observed by transmission electron microscopy. (C,D) Different regions of the spinal cord at 2 dpt; (C) central canal (cc) next to the injured site (black arrowheads); (D) extracellular matrix and red blood cells (red shadow) in the injury site. (E) Semithin section at 6 dpt. (F,G) Different regions of the spinal cord at 6 dpt; (F) ependymal cells in the rostral stump (black arrowheads); (G) macrophage (blue line) engulfing red blood cells (red shadow) in the cc. (H) Semithin section of the caudal stump at 10 dpt. (I-L) Different regions of the spinal cord at 10 dpt; (I) ependymal cells near to the injured site; (J) mitochondria (white arrowhead) in the apical surface of ependymal cell (blue line) in contact with the cc; (K) glial cell processes next to the injured site (white arrowheads); (L) intermediate filaments (white arrowheads) in the
glial process (green line). (M) Semithin section at 20 dpt. (N-P) Different regions of the spinal cord at 20 dpt; (N) ablation gap (red lines) filled with fibroblast-like cell (white arrowhead), and surrounded by extracellular matrix; (O) microglial-like cell (cyan line) with abundant rough endoplasmic reticulum (white arrowheads); (P) abundant collagen (col) fibers (dots) in the injured site. The red dotted lines indicate the injured site (A, B, E, H, M).
Glial cell and extracellular matrix response to spinal cord injury in R-Stage and NR-Stage. (A-C) Immunostaining against vimentin in (A) uninjured, and at (B) 2 and (C) 6 dpt from animals at NF stage 50. (D) Western blot against vimentin (VIM) and GAPDH of spinal cords samples obtained from uninjured (ui), and at 2, 6, 10 and 20 dpt in animals at NF stage 50. (E-G) Immunostaining against vimentin in uninjured (E-E'), and at (F, F') 10 and (G-G') 20 dpt from animals at NF stage 66. (H) Western blot against vimentin (VIM) and GAPDH of spinal cords samples obtained from uninjured (ui), and at 2, 6, 10 and 20 dpt in animals at NF stage 66. (I-O) Immunofluorescence against fibronectin in (I) uninjured, (J) 6 dpt, and (K) 10 dpt in NF stage 50; and (L) uninjured, (M) 10 dpt, and (N) 30 dpt in animals at NF stage 66. (O-P) Immunofluorescence against CSPG in (O) uninjured, and (P-P') at 40 dpt in NF stage 66. (Q-V) AFOG staining shown collagen (blue), cells (orange) and fibrin (red) in (Q) uninjured, and at (R) 6 and (S) 10 dpt in animals at NF stage 50, and in (T-T') uninjured, and at (U-U') 10 and (V-V') 20 dpt from animals at NF stage 66. (W) Analysis of gene expression change upon spinal cord injury comparing injured animals (Ts) with control sham (sham) surgery at 1, 2 and 6 days after injury in NF stage 50 (1R, 2R and 6R), and NF stage 66 (1NR, 2 NR and 6 NR). Colored and crosses scale indicates the level of increase upon injury in green (+, ++, ++++) and decrease in red (-, --, ---), data obtained from a previous RNAseq analysis (Lee-Liu, et al., 2014). The red dotted lines (B, F, J, M, N, P, R, U, V) and yellow arrows (C, K, S) indicate the injured site. Nuclei stained with Hoechst in blue (A-C; E-G; I-P).
Zebrafish regulatory regions of GFAP drive expression of EGFP in neural stem and progenitor cells, and astrocytes in Xenopus laevis spinal cord. (A-C) Lateral view of EGFP expression in the central nervous system at (A,B) NF-Stage 43, and (C) NF-Stage 50. (A) EGFP expression in the eye, brain and spinal cord (arrowheads). (B) EGFP/brightfield merge. (C) Dorsal view of EGFP expression in the optic tectum, hindbrain and spinal cord at NF stage 50. (D-F) Double staining against (D) EGFP and (E) Sox2. Panels

Figure 4
(F) showed merge image, and (F', F'') magnifications of the dorsal and ventral cells surrounding the central canal. (G-O') Characterization of EGFP cells by double staining at NF stage 66. (G-I'') EGFP and Sox2; (J-L') EGFP and Brain lipid-binding protein (BLBP); and (M-O') EGFP and Glutamine Synthase (GS). Nuclei are labeled in blue with Hoechst. (P-Q) Immunogold staining against EGFP at NF stage 50. (P) EGFP+ cell in contact with the central canal. (P') Magnification of square in P. Expression of EGFP is visualized by the black dots of the gold staining. (P'') Magnification of square in P'. Gold staining (black arrowhead) in close apposition with filaments (white arrowhead). (Q) Endfeet from an EGFP+ cell (colored green) in close contact with blood vessel (colored red). (R) Gene ontology analysis of the RNAseq from EGFP+ cells revealed the stem cell/neural precursor cell identity of these cells. (S) Dendrogram of EGFP+ cells and EGFP- cells showing the hierarchical clustering of EGFP+ cells with astrocytes and EGFP- cells with neurons and oligodendrocytes. Scale bar: C, F'-F'': 20 µm; A-B, D-F, I'-I'', L', O': 50 µm; G-I, J-L, M-O: 200 µm
Figure 5

Response to injury of Neural Stem and Progenitor Cells (A) Scheme of EdU treatment. (B) Click-iT staining for EdU (red), and immunofluorescence against EGFP (green), merge with nuclei (blue) in sham control animals at 2 days post sham operation (dps), and 2 dpt. (C) Graph of EdU-EGFP positive cells per mm3 at 2 dps (red bar) and 2 dpt (green bar). t-Test, ***: p<0.001. (D,E,F,G,H,I) Immunofluorescence against EGFP (green) at NF stage 50 in (D) uninjured, (E) 2 days post resection (dpr), (F) 6 dpr, (G) 7 dpr, (H) 10 dpr, and (I) 20 dpr. Magnifications are shown in panels D'-D'', E'-E'', F'-F'', G', H' and I'-I''. (J,K,L,M,N,O). Serial sections
from the same preparation shown in panels D,E,F,G,H,I double stained for EGFP (green) with the neuronal marker acetylated tubulin (red), and merge (orange). Nuclei are labeled in blue with Hoechst. White arrowhead highlights colocalization. Scale bar: D,E,F,G,H,I: 200 µm; D’-D'', E’-E'', F’-F'', G’, H’, I’-I'', J-J'', K-K'', L-L'', M-M'', N-N'', O-O'': 50 µm.

Figure 6
Cell Fate analysis of Neural Stem Progenitor Cells in response to spinal cord injury (A) Diagram of the experimental procedure. (B-M) Graphs of the ratio in the mRNA levels for the indicated genes between the EGFP+ cells and EGFP- cells in uninjured (ui), 2 and 6 dpt. (B) EGFP, (C-D) NSPC markers: (C) sox2, (D) nestin. (E-I) neuronal precursor/neurogenic differentiation markers: (E) achaete-scute homolog 1 (Ascl1), (F) neurogenin2a (neurog2a), (G) neurogenin3 (neurog3), (H) neuroD1, (I) doublecortin (Dcx). (J-K) Astrocytes markers: (J) vimentin-a (vim-a), (K) Aldh1l1. (L-M) Oligodendrocytes markers: (L) Sox10, (M) myelin basic protein (Mbp). n=2-3 samples. Standard error bar is included in each graph.

Figure 7

Ablation of NSPC blocks spinal cord regeneration (A) Diagram of the treatment of zGFAP::mCherry-NTR transgenic animals with metronidazol (MTZ) or vehicle, followed by spinal cord resection, swimming recording, and histological analysis. (B-E) Eye imaging (B,D) before treatment, and 7 days after incubation with (C) vehicle or (E) MTZ. (F,G) Spinal cord sections showing mCherry expression (F) before, and (G) 7 days after MTZ treatment. (H) Graph of swimming at 1, 10, 15, 25 dpr in sham (Sh) operated animals treated with MTZ (Sh-MTZ, blue boxes), and resected (Rs) animals incubated with vehicle (Rs-Vehicle, red boxes) or MTZ (Rs-MTZ, green boxes). (I-L) Immunofluorescence against Sox2 (green) and nuclei stained with Hoechst of spinal cord sections obtained from animals at 30 dpr from the (I,J) Rs-
vehicle, and (K,L) Rs-MTZ treated groups. Statistics in graph H: ANOVA-one way with Bonferroni post-test, ** p<0.01, n=4.

**Supplementary Files**

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