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TITLE:

Dynamic cell interactions allow spinal cord regeneration in zebrafish

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HIGHLIGHTS

- Diverse cell types promote spinal cord regeneration in zebrafish.
- Cell types in the injury site are highly dynamic and interactive.
- Pro-regenerative signals are discovered in zebrafish.
- Novel techniques transform discovery in zebrafish regeneration research.

ABSTRACT:

A spinal injury leads to complex reactions by glial, immune and other non-neural cells. In mammals these reactions are thought to prevent regeneration of axons and neuronal replacement. Here we review recent insights into how different cell types may cooperate to allow functional regeneration in zebrafish.

KEYWORDS:

Macrophages, oligodendrocytes, microglia, T-Cells, fibroblasts, Ependymo-radial glial cells

MAIN TEXT

After spinal injury in mammals, axonal connections are severed and neurons in the vicinity of the injury site show extensive cell death [1]. Axons and neurons are not replaced, leading to lifelong disability [2,3]. Zebrafish are initially paralysed after spinal injury, but regain neurons [4], axons [5] and swimming function [6] within weeks after injury. Apparently, the complex interactions that occur after spinal injury between glial cells, such as astrocytes and microglia, the innate and adaptive immune system and other cell types, such as fibroblasts, lead to net outcomes that inhibit regeneration in mammals, but facilitate it in zebrafish (Fig. 1A). Here we discuss recent insights into the cell types and associated molecules that facilitate successful spinal cord regeneration in zebrafish (summarised in Table 1).

Astroglia

In mammals, glial fibrillary acidic protein (GFAP) expressing astrocytic processes swell and produce inhibitory molecules, such as chondroitin sulfate proteoglycans, at the injury site. This so-called astrocytic scar may prevent axons from regrowing, but is also beneficial to recovery by preventing excessive secondary damage [7]. In zebrafish, astrocytic functions are performed by ependymo-radial glia cells (ERGs, also known as radial glia [8]), described below. However, after injury, “free” astrocyte-like cells appear. These astroglial-like cells in the injury site elongate in the direction of axon growth, such that they may form an astroglial bridge for axons to grow over [9]. However, in live observations in lesioned larvae with transgenetically labelled glia and neurons, axons mostly preceded glial growth. Moreover, in selective ablation experiments of GFAP-expressing glia using the nitroreductase system in larvae, axons bridged the injury site unimpededly [10]. Previous electron-microscopic observations indicate axonal processes as the first to cross a spinal injury site in adult goldfish [11]. This suggests that regenerating axons, and in particular pioneering axons, can follow different substrates than glial processes.

Astroglia-like cells promote regeneration by producing growth factors. For example, astroglia-like cells in the injury site of adult zebrafish are a source for connective tissue growth factor (Ctgf). Mutation and over-expression of the gene has shown that Ctgf promotes axonal regeneration, glial proliferation and functional recovery [12]. Ctgf may act on axons independently of the presence of glia. Glial cells also produce fibroblast growth factors, e.g. Fgf3, that support axon crossing, glial proliferation and elongation in adult zebrafish [9,13]. Hence, elongated astroglial-like cells in zebrafish support regeneration by not presenting a barrier to axon growth and by secreting growth factors.

Ependymo-radial glia

ERGs are the likely source of astroglia-like cells. In fact, ERGs are a hybrid form of ependymal cells and astrocytes with a progenitor cell function in adult and larval zebrafish [14-16]. ERGs also generate new neurons in the injured spinal cord. Similar ventricular stem cells exist in mammals [17,18], but they only contribute cells to the glial scar [19]. The new neurons in adult zebrafish are derived from different ventricular domains of ERGs that are reminiscent of progenitor domains in the developing neural tube and new neurons are integrated into the spinal network [20]. Regenerative neurogenesis in adult zebrafish is promoted by a number of developmental signals, such as Shh [21] or Fgf3 [9,13]. Dopamine [22] and serotonin

[23] derived from descending axons, as well as Ctgf, derived from reactive ERGs and other cells [12], also promote regenerative neurogenesis. ERGs react to immune signals with enhanced neurogenesis in the larval spinal cord [24] and other CNS regions of adults [25,26]. In summary, ERGs support regeneration by integrating a multitude of developmental and regeneration-specific signals to produce new neurons and growth-promoting astroglial-like cells.

Oligodendrocytes

Oligodendrocytes, which form the protective myelin sheaths for axons, interfere with regeneration in two ways in mammals. After injury, the break-down products of injured myelin sheaths (myelin debris) contain inhibitors of axonal regeneration, such as Nogo-A [27]. Moreover, injury leads to loss of myelin sheaths on intact axons, and insufficient regrowth of myelin sheaths (remyelination). This leaves axons vulnerable to degradation.

In zebrafish, the *Nogo* homolog *rtn4b* has a similar domain structure to its mammalian counterpart, but the most inhibitory domain of the protein (delta20) is less inhibitory in cross-species *in vitro* assays for the zebrafish protein compared to the mammalian version of the protein [28]. At the same time, zebrafish oligodendrocytes produce axon-growth promoting cell surface molecules, such as *mpz* [29] and *11cam* [30], at increased levels after injury. Mammalian oligodendrocytes do not detectably express these molecules. This means that myelin debris in zebrafish is less inhibitory than in mammals.

Moreover, the fish CNS has a very high capacity for remyelination, e.g. after experimental removal of myelin in the optic nerve. New myelin sheaths may be generated by new or pre-existing oligodendrocytes in larvae [31] or adults [32]. Interestingly, with age this remyelination capacity fails in adult zebrafish. Indeed, this correlates with reduced regenerative capacity of axons in aged adult zebrafish [33]. Hence, oligodendrocytes in fish contribute to successful regeneration by not inhibiting axon growth and by remyelinating axons.

Schwann cells

Schwann cells, the myelin-forming cells of the peripheral nervous system, dedifferentiate after peripheral nerve damage and promote regeneration in mammals [34]. Schwann cells invade a lesion site in mammals to a limited degree and have also been found to re-myelinate axons in the injured spinal cord of adult zebrafish [4], where they may promote axon growth and protect axons. However, many regenerated axons remain unmyelinated even after return of swimming function. Hence, Schwann cells may have a minor regeneration-promoting role after spinal injury in zebrafish.

Microglia

Microglia, the brain-resident macrophage population, react to an injury by rounding off and increasing in number after spinal injury in adult zebrafish [35], which is reminiscent of the amoeboid state of activated microglia in mammals [36]. While in mammals, the immune reaction is generally considered mal-adaptive [37], the microglia reaction promotes regenerative neurogenesis in zebrafish. Using dexamethasone to inhibit the immune reaction led to reduced regeneration of neurons in the injured larval spinal cord [24]. Similarly, neuronal regeneration after stab lesion of the telencephalon [26] and ablation of dopaminergic neurons [25] in the diencephalon of adult zebrafish can also be suppressed by dexamethasone. The

leukotriene C4 has been identified as possible signalling molecule from the microglial cells [26]. Interestingly, specific lack of microglia in *csf1ra/b* double-mutant larvae did not impair axonal regeneration in spinal-lesioned larvae [38]. Hence, microglia promotes neurogenesis after CNS injury in zebrafish, but may not be essential for axon regrowth.

Macrophages

A spinal injury site is invaded by blood-derived macrophages (Fig. 1B). In mammals, these may contribute to a prolonged inflammatory response that inhibits regeneration [37]. In larval zebrafish, the pro-inflammatory phase, indicated by high *il-1b* and *tnf* expression is rapidly ended and anti-inflammatory cytokines, such as *tgfb1a* and *tgfb3*, dominate [38]. However, in the absence of macrophages in the *irf8* mutant, inflammation is prolonged, as in mammals, and axonal regeneration is inhibited in lesioned larvae. Prolonged inflammation in these mutants is indicated by increased numbers of *il-1b* expressing neutrophils, underscoring the interactive nature of the immune response.

Axonal regeneration in *irf8* mutant larvae is almost completely rescued by inhibiting *Il-1b* function, indicating a key role of macrophages in controlling *Il-1b* driven inflammation and thus promoting axon regrowth. Interestingly, inhibiting *Tnf* signalling impairs axonal regeneration in wildtype larvae, suggesting a pro-regenerative role for this macrophage-derived pro-inflammatory cytokine [38].

How these macrophage-derived signals influence regenerative neurogenesis is not well understood. However, *Tnf*, released from dying neurons in the injured retina of adult zebrafish, alters expression of regeneration-related genes in Müller cells, the resident progenitor cells of the retina, and promotes neuronal regeneration [39]. Hence macrophages in zebrafish promote axonal regeneration by controlling the inflammation and may promote neurogenesis with specific signals after spinal injury.

Neutrophils

Neutrophils are early responders to a spinal injury (Fig. 1B) and are mostly thought to exacerbate the outcome in mammals [40]. Indeed, neutrophils express *il-1b* and their number is increased in the absence of macrophages after spinal cord injury in larval zebrafish [38]. This would make these cells anti-regenerative. However, timing experimental *Il-1b* inhibition such that only the early, neutrophil-related, peak is suppressed in wildtype larvae impairs regeneration [38]. Hence, the early neutrophil-mediated inflammation in zebrafish may kick-start axonal regeneration. A role for neutrophils in neuronal regeneration has not been clearly demonstrated.

Adaptive immune cells

Adaptive immune cells, such as T cells are thought to inhibit regeneration in mammals, as their depletion improves injury outcomes [41,42]. It has recently been shown that depletion of regulatory T cells in a spinal injury site in adult zebrafish strongly inhibits regeneration of axons, progenitor cell proliferation, addition of new neurons and functional recovery [43]. A possible mechanism is secretion of neurotrophin-3, a known axon growth factor, by the regulatory T cells in the spinal injury site. Remarkably, injury of other organs leads to secretion of other growth factors there, indicating a tissue context-dependent function of regulatory T cells. These results indicate a regeneration-promoting role of T-cells in zebrafish.

Fibroblasts

In mammals, fibroblasts are present in the center of the injury site to form a fibrotic scar [44], which, like the glial scar, produces extracellular matrix material [45]. Fibroblasts produce a number of extracellular matrix molecules, including collagens, thought to contribute to an inhibitory matrix [46].

In the injured larval zebrafish different types of fibroblasts enter the injury site. Some fibroblasts are responsive to injury-induced Wnt signalling, which promotes regeneration in larvae and adults [15,47]. There are also *pdgfrb*⁺ fibroblasts that produce Collagen XII [48]. *col12* expression depends on Wnt signalling. These types of fibroblasts are overlapping and there might be even more subtypes [10]. The origins of these populations need to be investigated. Possible sources are meningeal fibroblasts and *pdgfrb*⁺ pericytes. Regenerating axons grow along ColXII immunopositive longitudinal ECM fibrils and gain- and loss-of function experiments have demonstrated that ColXII promotes axonal regeneration in larval zebrafish. The lesion-site ECM is complex and is likely to contain more growth-promoting molecules. Whether fibroblasts can promote neuronal regeneration from spinal progenitor cells is unknown, however, ECM compounds are crucial for neurogenesis in other systems [49], making it possible that fibroblasts have a promoting role also for neuronal regeneration. Overall, fibroblasts are conducive to spinal cord regeneration in zebrafish.

Other cell types

There are additional cells in the injury site of mammals, such as pericytes [50] or endothelial cells [51], which contribute to modulation of the extracellular matrix environment. There is extensive cross talk between the nervous system and the vascular system during development [52,53]. Knock down of the melanoma adhesion molecule inhibits spinal cord regeneration in zebrafish, possibly by down-regulating vascular growth factors, but also inflammatory factors [54]. Additional lymphoid cells, such as B-cells, are also likely to modulate the immune response and thus spinal cord regeneration [55]. Hence, a large number of different cell types react to a spinal injury and, in zebrafish, support regeneration.

Conclusions and outlook

We have highlighted how different cell types that are activated by a spinal injury have beneficial influences on axonal and neuronal regeneration in zebrafish. The sum of these actions may well be sufficient to bring about the regenerative outcome after injury in zebrafish. However, another layer of complexity is added, if one considers that all of the activated cell types after injury are highly interactive. For example, the bi-phasic inflammatory response after injury involves control of neutrophil and keratinocyte expression of *il-1b* by macrophages [38]. It is likely that the immune reaction also alters reactions of other cell types, such as fibroblasts, e.g. in the deposition of different ECM components.

Novel techniques, such as single cell RNA sequencing, combined with genetic lineage tracing [56-58], will shed light on the diversity of cell types and on their interactions after injury by highlighting signalling molecules and changes in cellular phenotypes when other cell types are ablated or missing in mutants. The live-imaging opportunities that larvae offer [59], rapid somatic and stable germline gene mutation using CRISPR/Cas9 [60], and amenability to drug screening [61] will contribute to an accelerated discovery rate of complex cell interactions that promote

successful spinal cord regeneration in zebrafish. This knowledge will form the basis to understand the mis-communication between cell types that happens in the spinal injury site in mammals and will identify future therapeutic targets.

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AUTHOR CONTRIBUTIONS (CRediT nomenclature)

Writing - original draft: CGB, TB; writing - review & editing: CGB, TB

COMPETING INTEREST STATEMENT

Given their role as Guest Editors, Catherina and Thomas Becker had no involvement in the peer-review of this article and had no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Joseph Wu.

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TABLE

Table 1: How do different cell types promote spinal cord regeneration in zebrafish?

Cell type	factor/mechanism	axon regrowth	neurogenesis	citations
Astrocyte-like/ ERG*	Ctgf	+	+	[12]
	Wnt (?)	+	+	[47,62]
	Fgf3	+	+	[13]
	Shh	?	+	[21]
Descending axons	Dopamine	?	+	[22]
	Serotonin	?	+	[23]
Oligodendrocyte	Lack of Nogo	+	?	[28]
	Mpz	+	?	[29]
	L1cam	+	?	[48]
	Remyelination	+	n/a	[31,32]
Schwann cells	Remyelination	+	n/a	[5]
Microglia	?	-	+	[26,38]
Macrophages	Tnf	+	?	[38]
Neutrophils	early Il-1b	+	?	[38]
T-cells	Ntf3	+	+	[43]
Fibroblasts	ColXII	+	?	[10]

*given as one category, because the boundaries between these cell types are difficult to define.