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·Review·

Axonal regeneration after spinal cord injury in zebrafish and mammals: differences, similarities, translation

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Spinal cord injury (SCI) in mammals results in functional deficits that are mostly permanent due in part to the inability of severed axons to regenerate. Several types of growth-inhibitory molecules expressed at the injury site contribute to this regeneration failure. The responses of axons to these inhibitors vary greatly within and between organisms, reflecting axons' characteristic intrinsic propensity for regeneration. In the zebrafish (*Danio rerio*) many but not all axons exhibit successful regeneration after SCI. This review presents and compares the intrinsic and extrinsic determinants of axonal regeneration in the injured spinal cord in mammals and zebrafish. A better understanding of the molecules and molecular pathways underlying the remarkable individualism among neurons in mature zebrafish may support the development of therapies for SCI and their translation to the clinic.

Keywords: spinal cord injury; axonal regeneration; growth inhibition; functional recovery; zebrafish

Introduction

Spinal cord injury (SCI) in mammals typically results in permanent neurological deficits below the level of the lesion. Neurological deficits are primarily caused by the injury-induced interruption of ascending and descending axonal tracts and can be worsened by secondary injury-related events[1, 2]. In the central nervous system (CNS), the distal part of a severed axon undergoes Wallerian degeneration, while the proximal part typically retracts to some degree and forms a 'retraction bulb'. Some types of neurons are intrinsically unable to re-grow their axons after damage, while others do have some intrinsic growth ability[3, 4]. However, the regeneration attempts of the latter typically fail because, at least in part, of growth-inhibiting molecules in the milieu of the injury site. The balance between intrinsic and extrinsic molecular events determines the overall axonal regeneration response. A better understanding of this balance may support the development and clinical translation of effective therapies for SCI that involve axonal regeneration.

In contrast to the mammalian CNS, axons of embryonic neurons, axons in peripheral nervous system (PNS), and axons in some non-mammalian species regenerate after severance[5-7]. While all these models are useful for studying the mechanisms of axon regeneration, here we focus on the zebrafish (*Danio rerio*) due to the many recent studies that have elucidated some of the key molecules in this process.

In the adult zebrafish, many brainstem neurons are able to regenerate axons across an injury in the spinal cord and extend caudally[3]. This process is typically accompanied by anatomical restoration at the lesion site and impressive functional (swimming) recovery[4]. Interestingly, not all axons that normally project into the spinal cord in adult zebrafish regenerate after SCI. The mechanisms underlying this remarkable difference between zebrafish axons are incompletely understood. Comparison

of the molecules fundamental to this largely variable ability to regenerate axons may provide clues about which signaling pathways are involved.

The zebrafish gives us the unique opportunity to investigate the individual mechanisms underlying successful and failed axon regeneration while extrinsic parameters within CNS milieu remain constant. The sequencing of the zebrafish genome has provided several molecular tools (microarray, next-generation sequencing, transgenic animals, morpholinos) to perform functional studies that can verify the level of involvement of certain molecules and/or pathways in axonal regeneration. In addition, the transparency of zebrafish embryos, larvae, and the adult Casper strain, enables *in vivo* studies of axonal regeneration using two-photon microscopy, laser ablation, and Ca^{2+} imaging.

Axonal Regeneration in Mammals

Adult mammalian CNS axons do not spontaneously regenerate after a lesion. The mechanisms preventing successful regeneration can be environmental (extrinsic) or within the axon/neuron (intrinsic). Extrinsic mechanisms include the relative lack of growth-promoting molecules and/or the surplus of growth-inhibitory molecules expressed in the injury milieu. Intrinsic mechanisms involve molecules expressed within the neuron that limit or prevent regeneration.

Axonal Regeneration in Zebrafish

Adult zebrafish have 20 brainstem nuclei that project axons into the spinal cord[3]. Becker and collaborators found that after a complete transection at the level of the brainstem-spinal cord transition zone, 32–51% of neurons in the nucleus of the medial longitudinal fascicle (NMLF), magnocellular octaval nuclei, and intermediate reticular nuclei regenerate axons across the lesion and up to 4 mm into the caudal stump[8]. However, neurons in other brainstem nuclei that project into the spinal cord, such as the red nucleus, nucleus of the lateral lemniscus, and tangential nucleus, as well as Mauthner neurons and dorsal root ganglion neurons, fail to regenerate axons after SCI[9, 10].

Molecular Determinants of Axonal Regeneration

Animal experiments have led to the discovery of several intrinsic and extrinsic mechanisms responsible for the axonal regeneration response in the injured CNS.

Extrinsic Factors Involved in Axonal Regeneration in Mammals and Zebrafish

Axonal growth inhibitors bind to receptors on neurons and initiate a signaling cascade that leads to regeneration failure. There are two major sources of inhibitory molecules that prevent axonal regeneration in the injured mammalian spinal cord. Scar tissue at the injury site contains reactive astrocytes expressing growth-inhibitory chondroitin sulfate proteoglycans (CSPGs). Myelin debris at the site of injury expresses neurite outgrowth inhibitor A (Nogo-A or reticulon-4), myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Fig. 1)[11].

CSPGs The glial scar contains reactive astrocytes that create a physical barrier to axonal regeneration. In addition, reactive astrocytes secrete CSPGs, which are a diverse group of molecules comprised of a core proteoglycan with sulfated glycosaminoglycan (GAG) chains. In mammals, GAG chains bind to the receptor protein tyrosine phosphatase sigma (RPTPσ) and leukocyte common antigen-related phosphatase on neurons, resulting in the inhibition of axonal regeneration[12, 13]. Depolymerization of GAG chains by chondroitinase ABC or inhibition of their synthesis using DNA enzymes improves axonal regeneration that is accompanied by improved functional recovery in rats[14, 15]. In the normal, uninjured mammalian CNS, CSPGs with hyaluronan and link proteins form the perineuronal net which regulates synaptic stabilization[16]. Different types of mammalian neurons are differently inhibited by CSPGs *in vitro*[17] and the composition of perineuronal nets differs between neurons[16], resulting in variability among neurons in their ability to regenerate severed axons.

The SCI site in zebrafish differs markedly from that of mammals. In zebrafish, reactive astrocytes have not been observed. After a complete transection, ependymo-radial glial cells proliferate and extend across the lesion site thereby forming a ‘bridge’ between the spinal cord stumps[18]. While CSPGs are normally expressed in the zebrafish CNS, it is unknown whether their presence is increased after injury. However, during regeneration of the optic pathway, CSPGs and tenascin-R prevent axons from making aberrant connections with neurons in the pretectum, demonstrating the ability of zebrafish CSPGs to

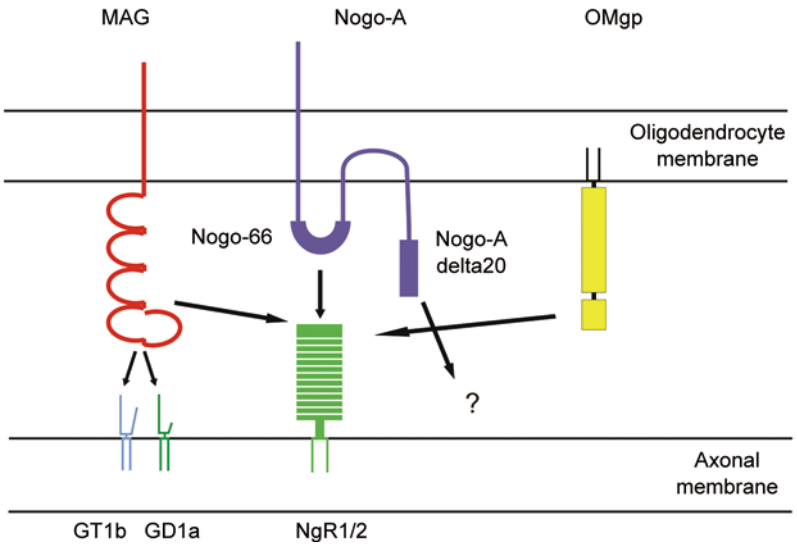

Fig. 1. Myelin inhibitors of axon regeneration. Myelin-associated glycoprotein (MAG) halts axonal regrowth by binding to the gangliosides GD1a or GT1b and/or Nogo receptors 2 and 1 (NgR 2/1). Neurite outgrowth inhibitor A (Nogo-A) contains two axonal outgrowth inhibitory domains, Nogo-66 and Nogo-A delta20. The receptor for Nogo-66 is NgR1, while the receptor for Nogo-A delta20 is unknown. Oligodendrocyte myelin glycoprotein (OMgp) inhibits axonal regrowth by binding to NgR as well.

inhibit axonal growth[19].

Nogo-A Nogo-A is a member of the reticulon family of proteins that are mainly found in the endoplasmic reticulum. Its C-terminus contains two transmembrane hydrophobic regions flanking an extracellular hydrophilic loop of 66 amino-acids (Nogo-66) that inhibits neurite outgrowth in mammals by binding to Nogo receptor 1. Its N-terminus contains another neurite outgrowth inhibitory domain termed Nogo-A delta20[20] for which the receptor is currently unknown. Nogo-A is expressed in oligodendrocytes, immature neurons, and adult neurons in the cortex, hippocampus, and dorsal root ganglia[21]. Neuronal Nogo-A plays a role in synaptic plasticity and protection against reactive oxygen species in mammals[22] and is involved in zebrafish PNS axon outgrowth and pathfinding[23]. Treatment with anti-Nogo-A antibody leads to axonal regeneration and collateral sprouting of spared axons in the rat and primate corticospinal and raphespinal tracts[24] and this has recently been tested in a Phase I clinical trial (anti-human Nogo-A antibody ATI355, Novartis Pharma, http://www.clinicaltrials.gov/ct/show/NCT00406016). Anti-Nogo-A treatment fails to improve the regeneration of ascending sensory axons in adult rats[25]. In zebrafish, Nogo-A lacks the N-terminus neurite inhibitory domain (i.e. Nogo-A delta20 in mammals). The Nogo-66 sequence in zebrafish, in contrast to mammals, is growth-permissive to both zebrafish and mouse axons *in vitro*[26].

MAG MAG is a sialic acid binding immunoglobulin (Ig)-like lectin (Siglec-4) composed of five IgG-like domains, a transmembrane domain and a shorter or longer cytoplasmic domain (S-MAG or L-MAG). The neuronal receptors for MAG are the gangliosides GD1a and GT1b and Nogo receptors 1 and 2 (NgR1 and NgR2)[27]. Recent *in vitro* experiments revealed the existence of cell-specific mechanisms involved in MAG-mediated inhibition of axonal regeneration. While cerebellar granule neurons respond to MAG mainly through ganglioside-dependent pathways, dorsal root ganglion neurons, hippocampal neurons, and retinal ganglion cells respond through NgR1/2[28, 29]. In zebrafish, MAG is present in myelin in three different splice variants and its cytoplasmic tail sequence is largely different from mammalian MAG, suggesting that it might have different intracellular signaling pathways[30]. The axonal outgrowth of cultured zebrafish spinal neurons is decreased in the presence of mammalian MAG, however similar experiments using zebrafish MAG have not been done yet. The same neurons have been shown to express NgR[31]. A study by Viljetic *et al.* showed that neurons in the granule cell layer of the zebrafish cerebellum express the gangliosides GD1a and GT1b, while Purkinje neurons do

not[32]. It is also worth mentioning that Schwann cells, which weakly or do not express Nogo-A and MAG, proliferate and remyelinate zebrafish CNS axons after SCI[4].

Although many *in vitro* and *in vivo* studies have shown the involvement of Nogo-A, MAG, and OMgp in axonal regeneration, there are still conflicting results. For instance, spinalized triple-mutant mice deficient in Nogo-A,B,C, MAG, and OMgp show neither enhanced axonal regeneration nor improved functional recovery[33]. It is possible that constitutive knockout mice have compensatory mechanisms that overcome such a triple deletion. However, inducible knockout mice may show enhanced axonal regeneration similar to pharmacological inhibition of these inhibitors. Further studies of the physiological and developmental roles of these molecules are needed to elucidate their functions in the CNS.

A direct comparison of mammalian and zebrafish extrinsic factors involved in axonal regeneration indicates that the CNS in zebrafish is more permissive to axonal growth. Still, even with a relatively permissive environment, more than half of zebrafish spinal axons do not regenerate after SCI. This suggests that the difference in axonal regeneration ability between non-regenerating and regenerating axons in the adult zebrafish lies in their intrinsic propensity for axonal growth.

Intrinsic Factors Involved in Axonal Regeneration in Mammals and Zebrafish

When presented with a permissive growth substrate such as a Schwann cell-containing peripheral nerve graft or embryonic nervous tissue, certain CNS neurons fail to regenerate damaged axons, while others regenerate to some degree. For instance, Purkinje neurons, but not inferior olive neurons, are unable to regenerate axons into an embryonic nervous tissue graft[34]. Another study showed that Purkinje neurons also fail to regenerate axons into a peripheral nerve graft while neurons of the reticular nucleus in the thalamus are able to do so[35]. Clearly, axons are intrinsically predetermined to grow or not after an injury. Several key molecules determine this intrinsic axon growth ability.

Growth-associated protein-43 (GAP-43) GAP-43 is a cytoplasmic protein that can be inserted into membrane by palmytoil anchors on cysteines 3 and 4. It is a well-characterized protein that has been associated with successful axonal regeneration in different types of neurons and in different species, including mammals[36]. It is localized in axonal terminals, and highly expressed during CNS development, PNS regeneration, and the regeneration of mammalian CNS axons into permissive grafts (Fig. 2). In the zebrafish, brainstem nuclei with high regenerative capacity express GAP-43 in 84–92% of their neurons at 6 days post-SCI, while nuclei with low regenerative capacity express GAP-43 in 49–63% of their neurons. In the latter nuclei, GAP-43 expression decreases dramatically within 2–3 weeks post-injury[8, 10].

L1 cell adhesion molecule (L1CAM) L1CAM is an axonal transmembrane protein with an extracellular domain that contains several immunoglobulin-like domains and fibronectin-like repeats (type III). L1CAM is important for

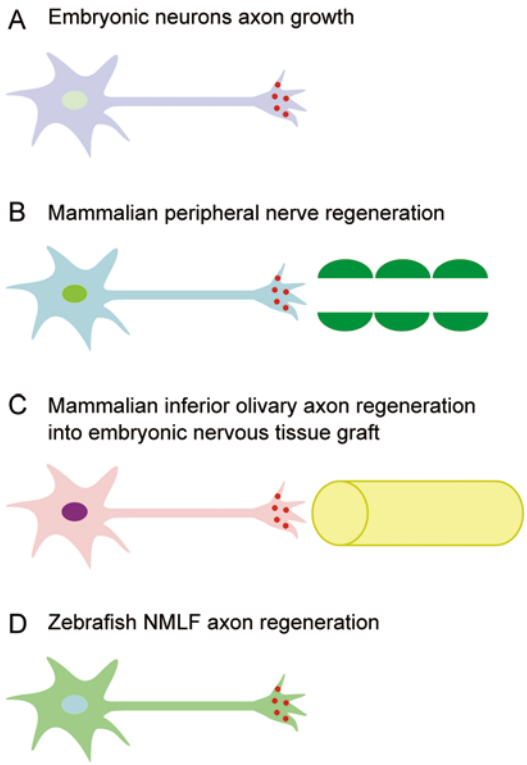

Fig. 2. Growth-associated protein 43 (GAP-43) is expressed in different types of neurons during successful axonal outgrowth. GAP-43 (red circles) is expressed in growth cones of: mammalian embryonic neurons *in vivo* and *in vitro* (A), mammalian peripheral nerve axons during regeneration into a Schwann cell canal (green semi-ovals) (B), mammalian inferior olivary axons during regeneration into an embryonic nervous tissue graft (yellow cylinder) (C), and zebrafish nucleus of the medial longitudinal fascicle (NMLF) axons during regeneration after spinal cord injury (D).

CNS development, neuronal migration and differentiation, and axonal regeneration[37, 38]. In zebrafish, there are two homologs of L1: L1.1 and L1.2. L1.1 is expressed in 84–86% of neurons in nuclei of high regenerative capacity and in 40% of neurons in nuclei of low regenerative capacity[8-10].

Cysteine- and glycine-rich protein 1a (CRP1) In a recent study, Ma and colleagues performed a temporal analysis using a microarray on the NMLF dissected by laser-capture from uninjured and SCI zebrafish[39] and showed increased expression of *crp1* mRNA after SCI. Several other mRNAs were also increased: matrix metalloproteinase-9 (*mmp9*), suppressor of cytokine signaling 3b (*socs3b*), *gap43*, *contactin-2* and major vault protein (*mvp*). Quantitative-PCR and *in situ* hybridization validated the increased expression of *crp1* at 11 days post-SCI. In addition, using two different morpholino-knockdown approaches by which the protein expression of CRP1 was decreased, the researchers found impaired swimming recovery and decreased number of axons that regenerated[39]. CRP1 is also known to be up-regulated during optic nerve regeneration in zebrafish[40]. The function of CRP1 appears to be in actin bundling[41] and its expression is high in the filopodia of cultured mammalian hippocampal neurons[42].

Contactin-2 Contactin-2 (axonin-1, transient axonal glycoprotein, TAG-1) is a glycosylphosphatidylinositol-anchored protein that is either membrane-bound or secreted into the cerebrospinal fluid. The membrane-bound contactin-2 functions as a cell-adhesion molecule, while the soluble form promotes neurite outgrowth from cultured mammalian neurons[43]. Its expression in adult mouse brain is restricted to the olfactory bulb and hippocampus, which suggests that it might play a role in plasticity-related events within the CNS[44]. Microarray revealed increased expression of *contactin-2* mRNA in zebrafish NMLF neurons after SCI[38], which was further verified by qPCR and morpholino-mediated knockdown of *contactin-2* mRNA, which caused decreased bulbo-spinal axonal regeneration[45].

Suppressor of cytokine signaling 3 (SOCS3) Deletion of SOCS3 in adult mouse retinal ganglion neurons improves axonal regeneration[46]. SOCS3 negatively regulates the gp130-dependent JAK/STAT pathway, and when gp130 is deleted the effects of SOCS3 deletion are abolished, indicating that SOCS3 is a major intrinsic mechanism that limits successful axonal regeneration. Interestingly, microarray analysis by Ma and colleagues showed that expression of *socs3b* mRNA is increased in the NMLF after SCI in zebrafish. So far functional studies have not been performed[39].

PI3K/AKT/mTOR pathway Recently, it was demonstrated that deletion of the phosphatase and tensin homolog (PTEN) gene improves axonal regeneration and prevents apoptosis in mouse retinal ganglion neurons. PTEN is a known inhibitor of the PI3K/AKT/mTOR pathway. Direct inhibition of mTOR by rapamycin abolishes the effects of PTEN deletion in retinal ganglion neurons[47]. *In vivo* experiments have shown that PTEN deletion leads to sprouting and regeneration in the mouse corticospinal tract and dorsal root ganglion neurons[48, 49]. During development, the expression of mTOR decreases in most retinal ganglion neurons, and axotomy further decreases its expression, suggesting that inactivation of mTOR is a major intrinsic mechanism that limits axonal regeneration. One of the functions of mTOR is the regulation of protein synthesis which is needed after axotomy to synthesize new organelles (cytoskeleton) in the extending axon. In zebrafish, the functions of PTEN and mTOR in axonal regeneration have not been studied.

Major vault protein (MVP) MVP is part of a vault cytoplasmic ribonucleoprotein, which is an organelle with a somewhat elusive function in eukaryotic cells. The vaults are involved in the transport of mRNA from nucleus to cytoplasm[50]. MVP accounts for 75% of the total vault complex which contains small un-translated vault RNA, telomerase-associated protein-1 and vault poly (ADP-ribose) polymerase. In zebrafish spinal cord, the expression of MVP mRNA and protein increases at six and 11 days post-SCI as evidenced by microarray, qPCR and immunohistochemistry. Knockdown by morpholino against *mvp* mRNA decreased MVP protein expression in spinal cord tissue, impaired swimming recovery, reduced bulbo-spinal axonal regeneration, and caused fewer synapses below the SCI site[51]. MVP is up-regulated in dorsal root ganglion neurons after spinal nerve ligation in rats[52] as well as in neurons of the electric ray following injury[53]. MVP has been shown to regulate the PI3K/Akt and JAK–STAT pathways in cancer cells[54, 55]. These pathways are involved in the intrinsic inhibition of axonal regeneration in mammals (see above). It would be of great interest to investigate whether MVP influences these pathways during axonal

regeneration in the zebrafish after SCI.

miR-133b MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression by modifying translation and mRNA degradation. A single miRNA can have multiple target mRNAs and a single mRNA can have multiple targeting miRNAs. Recently, Yu *et al.* found that *miR-133b* is expressed in the NMLF, superior reticular formation, intermediate reticular formation, magnocellular octaval nuclei and spinal grey matter in normal zebrafish[56]. At one and seven days post-SCI, the number of NMLF neurons expressing *miR-133b* increases 5–6 times as compared to sham-injured fish. Morpholino-mediated knockdown of *miR-133b* expression decreases the number of NMLF neurons exhibiting axonal regeneration and increases RhoA expression. In rat, *miR-133b* expression is up-regulated at 4 h but dramatically decreases at one day post-SCI[57]. There are several targets in the mammalian CNS for *miR-133b* including RhoA[58], calcineurin[59] and caspase-9[60] which play different roles in regeneration. Rho GTPase is involved in axonal growth inhibition; a RhoA inhibitor is currently being tested in phase I/IIa clinical trials for SCI[61]. Calcineurin and caspase-9 are pro-apoptotic molecules and their inhibition results in improved functional recovery after spinal cord and brain trauma[62, 63].

Axonal regeneration in the adult mammalian CNS is limited even when certain inhibitors of axonal regrowth are removed. Study is further complicated by the fact that not all neurons respond the same way to treatment. The zebrafish offers a unique opportunity to study the differences between successful and unsuccessful axonal regeneration while the extrinsic factors are constant. The expression of GAP-43 and L1CAM is higher in highly-regenerative brainstem nuclei in zebrafish than in low-regenerative nuclei, which is similar to certain mammalian neurons presented with a permissive substrate. Recent studies in zebrafish have revealed several new intrinsic factors involved in successful axonal regeneration, such as CRP1, contactin-2, MVP and *miR-133b*, whose functions in mammalian neurons need to be further investigated.

Lessons Learned from Zebrafish That Can Be Applied to Mammalian SCI

A typical spinal cord contusion damages axonal pathways originating from different nuclei in the brain. The neurons vary in how their signaling pathways respond to axonal growth-inhibitors in the injury milieu[28, 29] and have different, unique, intrinsic axonal growth properties[34, 35] (Fig. 3). A better understanding of the individual characteristics of neurons with regard to their regenerative propensity may point at the necessity for interventions in more common mechanisms or multiple interventions in separate mechanisms to achieve significant axonal regeneration and thus functional restoration after SCI. Studies of axonal regeneration-competent organisms such as zebrafish contribute to elucidating common and unique molecules and molecular pathways involved in successful and failed axonal regeneration after SCI.

About 30–50% of brainstem neurons in zebrafish regenerate axons after a complete spinal cord transection and this results in basic locomotor (swimming) recovery.

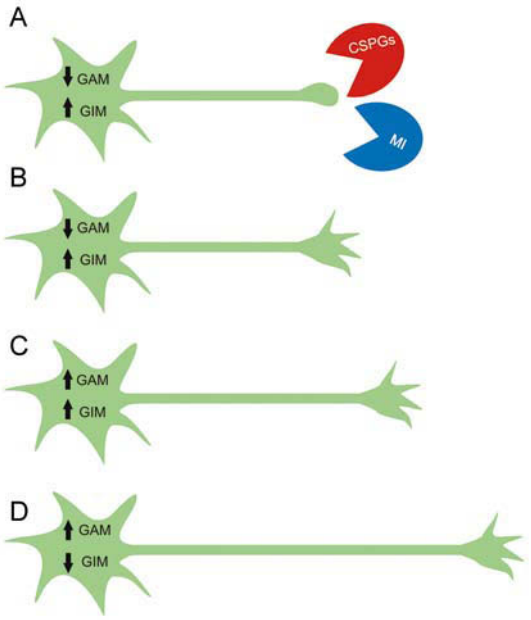

Fig. 3. Differential mechanisms involved in axonal regeneration. Most axons are inhibited from regenerating by a combination of extrinsic and intrinsic factors (A). Elimination of extrinsic inhibitors alone is sufficient to promote regeneration of certain axons (B), while others require up-regulation of growth-associated molecules (GAMs) (C) or a combination of up-regulation of GAMs and down-regulation of growth-inhibitory molecules (GIMs) along with elimination of extrinsic inhibitors for successful regeneration (D). CSPGs, chondroitin-sulfate proteoglycans; MI, myelin inhibitors of axonal regeneration.

Most axons, including ascending axons, fail or do not attempt to regenerate. Comprehensive answers about what is necessary for functional repair and if there is any reorganization of neuronal circuitry that contributes to functional repair will most likely come from a better understanding of the mechanisms crucial for regeneration as well as those for failed and no regeneration. Studies revealing the gene and protein expression profiles in axon populations with different regenerative responses following SCI are important in providing target genes/molecules for the design of future therapies.

The zebrafish is an excellent model in which to study the mechanisms underlying successful and failed axonal regeneration after SCI. Due to embryo transparency, the zebrafish has been established as a model of vertebrate development for several decades and sequencing of its genome has made many molecular tools available, including morpholinos, transgenic fish, and microarrays/next-generation sequencing. A recent study by Goldshmit and colleagues used different techniques, including transgenic zebrafish and time-lapse imaging, to study the influence of fibroblast growth factor signaling on the formation of the glial cell bridge and its association with regenerating axons[18]. This is a nice example of how currently-available zebrafish research tools can help in unraveling the mechanisms that drive successful anatomical recovery after SCI. Similar tools can be used to discover mechanisms that determine the individual characteristics of zebrafish neurons in their propensity for axonal regeneration after CNS injury as well as the extent of functional repair controlled by supraspinal pathways. Subsequently, such information may lead to an improved understanding of axonal regeneration responses in the mammalian spinal cord and serve to develop effective therapies for SCI.

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