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Review article

Glial cell ecology in zebrafish development and regeneration

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ABSTRACT

Zebrafish have been found to be the premier model organism in biological and biomedical research, specifically offering many advantages in developmental biology and genetics. The zebrafish (*Danio rerio*) has the ability to regenerate its spinal cord after injury. However, the complete molecular and cellular mechanisms behind glial bridge formation in zebrafish remains unclear. In our review paper, we examine the extracellular and intracellular molecular signaling factors that control zebrafish glial cell bridging and glial cell development in the forebrain. The interplay between initiating and terminating molecular feedback cycles deserve future investigations during glial cell growth, movement, and differentiation.

1. Introduction

Zebrafish, unlike humans, have the capacity to regenerate their spinal cord after injury [1, 2, 3]. Glia are crucial in the context of development, disease progression, and injury response [4, 5]. In addition, axonal regeneration is an important step during spinal cord regeneration, and there are many excellent review articles on this important topic [6, 7, 8, 9, 10, 11, 12, 13]. In our review article, we wish to focus more attention on how extracellular and intracellular molecular signaling are integrated to control glial bridge formation after spinal cord injury [1]. In addition, we will focus our review on ependymal cells, radial glia, and astroglia, but not oligodendrocytes, cell types in zebrafish spinal cord regeneration. After a wave of glial cell proliferation, glial cells migration and differentiate into glial cell bipolar morphology where "polar bases" of glial cells form across from each other at the lesion site [1]. Glial cell bridging is characterized by mature glial elongating over the lesion to form glial bridges. These phases and transition points in glial cell development and repair over space and time are defined as *glial cell ecology* (Table 1, Figure 1). Here, we use the term 'cell ecology' to describe the relationship of glial cell types within its cellular surroundings, or micro-environment during physiologic or regenerative conditions.

Over the past few decades, there have been important investigations into the cellular mechanisms in response to spinal cord injury [2, 14, 15, 16, 17]. For instance, spinal cord injury studies in goldfish using immunostaining method demonstrate trailing astroglial cells that appear behind the regenerating axon at the site of injury [16]. Another study by Wehner and colleagues demonstrated that the ability for axon cell function and axonogenesis is dependent on Wnt/β-catenin induced Collagen XIII in fibroblast-like cells at the site of injury in zebrafish [18]. In addition, Kato et al. and Losada-Perez and colleagues collectively offered novel insights into the mechanisms of glial cell specification and differentiation mechanisms in response to neuronal injury in the fruit fly *Drosophila melanogaster* [19, 20]. While there are recent review articles that focus on fruit fly *Drosophila* mechanisms and spinal cord regeneration mechanisms in mammals [21], less is known regarding extracellular termination signals that control zebrafish glial bridge formation and how this can be harnessed for rational human spinal cord disease.

After reflection of the molecular and cellular events that occur during each phase of spinal cord regeneration, it is still unclear how each phase is controlled over time and space after the spinal cord injury; furthermore, it is unclear how cells integrate molecular cues and interpret them to proliferate, migrate, and communicate with other cell types, such as newly-differentiated axons and glial cells, during each phase. Molecular positive and negative feedback mechanisms help control cell homeostasis and control cell differentiation. For example, Hildago and Logan put forth positive feedback signaling or "go" signaling, and negative feedback or

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Table 1. Definition of terms in glial cell ecology.

Term	Definition
Glial cell ecology	Refers to the glial cell identity state based upon its gene expression and cell behavior at a given transition phase in time and space (or spatiotemporal window) within a given microenvironment of the nervous system during normal or pathogenic/injured condition. This concept of molecular and cell ecology can also be applied into different biological frameworks.
Microenvironment	Refers to the location of a given cell identity type within a tissue or nervous system at a given point in the life cycle of the organism.
Transition Point	For each phase in either normal development or response to injury, a cellular process is controlled by molecular signals that either promote or inhibit the phase for a given cellular process.

feedback inhibition or "stop" signaling framework to explain mechanisms of *Drosophila* glial regeneration. By extending the "go and stop" signals in *Drosophila* and mouse model organisms in cell growth and differentiation [21], we wish to provide a perspective on a feedback cycle mechanisms framework in which cells or its microenvironment provide a series of cycles between positive and termination signal waves during glial cell bridge formation (Figure 2A, Table 2). In the following sections of our review article, we will focus on the following mechanistic actions: (1) Fgf signaling and *ctgfa* during zebrafish glial cell bridge formation; (2) molecular action of axon guidance molecules and Wnt/β-catenin signaling in the zebrafish forebrain (Figure 2B, Table 2); and (3) the specific molecular action of Wnt inhibitor Dkk1 and glucocorticoid signaling through receptor during glial cell bridge formation upon zebrafish spinal cord injury (Figure 2A).

2. Molecular signaling during glial bridge formation after zebrafish spinal cord injury

In order to determine which molecular factors are required for glial cell bridge formation, a genome wide profiling screen for secreted factors upregulated during spinal cord regenerative was performed. It was found that connective tissue growth factor a (ctgfa) was expressed in and around glial cells in the initial events leading to glial bridge formation [2]. Loss-of-function ctgfa mutant resulted in disruptions to the spinal cord repair, while overexpression promoted regeneration after spinal cord injury. During this phase, it was found that fibroblast growth factor (Fgf) signaling is required for glial bridge formation [1]. Additionally, glial activation is regulated by Fgf signaling and loss of function Fgf resulted in inhibition of glial bridges, disrupting the bipolar component of glial bridging. Interestingly, delayed heat-shock induced inhibition of Fgf signaling led to a set of novel neuronal bipolar cells. It will be important in the future to unveil the cellular identity of these Fgf-signaling independent cell types during spinal cord regeneration and axonogenesis. Furthermore, the role of ctgfa in these Fgf signaling-independent cell types remains to be determined. Collectively, these studies also suggest that additional Fgf-independent signaling mechanisms are also likely involved, and some of these candidate signals include Notch and canonical Wnt signaling. Finally, in another study by Wehner and colleagues, Mtz prodrug induced GFAP + glial cell lineage ablation studies demonstrated that axonal bridges still form in the absence of GFAP + expressing glial. In addition, global inhibition of canonical Wnt/β-catenin signaling through overexpression of Wnt inhibitor Axin1, but not ependymal glial cell lineage, led to decreased number of glial bridge formation post-injury. In summary, Fgf signaling and ctgfa are required for glial bridge formation in zebrafish (Figure 2A). Future research directions in how Fgf signaling coordinates cell movement with glial bridge differentiation, and using cell transplantation studies to elucidate how Wnt/β-catenin upregulation of CollagenXIII acts in ctgfa + ependymal glial, and/or fibronectin+ and collagen1a2+ expressing fibroblast-like cells during zebrafish spinal cord regeneration warrant future investigation.

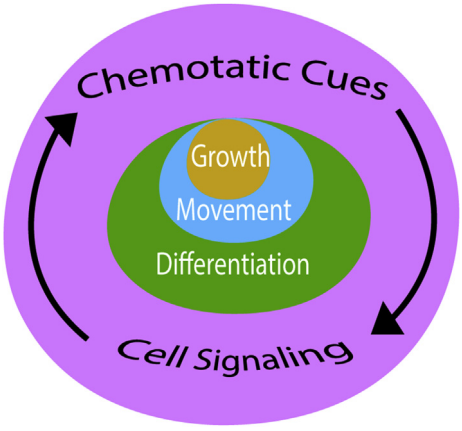

Figure 1. Cell Ecology Framework during Zebrafish Spinal Cord Regeneration. An ecological framework integrating extracellular chemotactic cues and cell signaling (magenta circle) that control growth (orange circle), cell movement (blue circle), and cell differentiation (green circle) during zebrafish spinal cord regeneration. Feedback mechanisms help maintain molecular and cellular homeostasis during regeneration (black arrows).

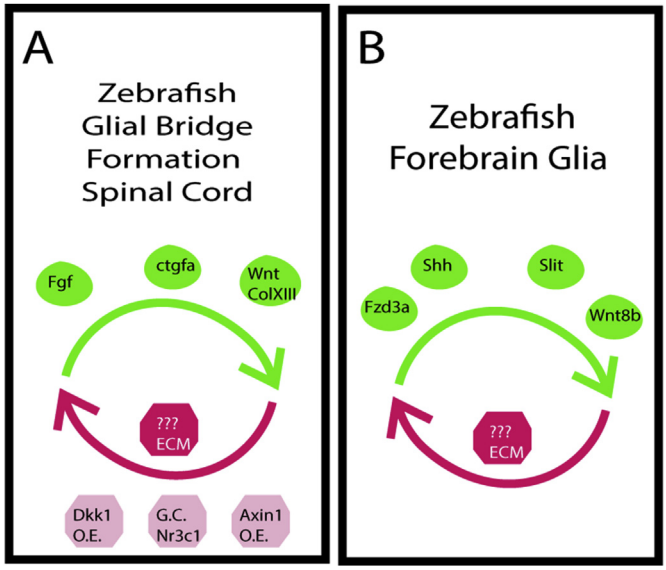

Figure 2. Feedback Cycles in Glial Cell Development and Regeneration. (**A-B**) Molecular positive and negative feedback cycles that govern zebrafish glial cell bridging in the spinal cord upon injury (**A**) and zebrafish glial cell connections in the developing zebrafish forebrain (**B**). Within each molecular feedback cycle, positive feedback arrow (green arrow) and negative feedback (or feedback inhibition) arrow (red arrow) are depicted for each process. Molecular positive feedback factors (green 'go' signal) and termination signals (red 'stop' signal) are indicated along with unknown factors listed as question marks. 'Go' signals include *ctgfa*, *Fgf*, and Wnt/β-catenin activating Collagen XIII (Wnt ColXIII). Dkk1 overexpression (Dkk1 O.E.), glucocorticoid signaling through receptor Nr3c1 (G.C. Nr3c1), and overexpression of Axin 1 (Axin 1 O.E.) are termination signal during zebrafish glial bridge formation in spinal cord in response to injury. Extracellular signals that act as termination factors remain area for future investigations (ECM red 'stop' signal).

Table 2. Highlighted signaling molecules involved in glial developmental processes and glial bridge formation.

Cellular Process	Developmental System	Associated Signaling Molecules	Model Organism	References
Initial Glial and Neural Injury Response	Ventral nerve cord (VNC) Kon expressing cells NG2+ OPC cell growth	NFkB Dorsal	*Drosophila* *Mus musculus*	Hidalgo and Logan (2017)
Cell Migration	To be identified and investigated	unknown	To be identified and investigated	
Cell Growth and Homeostasis	Ventral nerve cord (VNC) Kon expressing cells NG2+ OPC cell growth	NFkB/Dorsal Kon Notch *Notch1*	*Drosophila* *Mus musculus*	Hidalgo and Logan (2017)
Glial Bridge Cell Specification	To be identified and investigated	unknown	To be identified and investigated	
Glial Bridge Cell Differentiation	Ventral nerve cord (VNC) Kon expressing cells NG2+ OPC cell growth	Pros *Notch1*	*Drosophila* *Mus musculus*	Hidalgo and Logan (2017)
Glial Bridge Cell Formation	Zebrafish Spinal Cord regeneration Postoptic commissure (POC) Anterior Commisure (AC) optic nerve in central nervous system in zebrafish embryo Forebrain commissural plate Perineural glial bridge formation in peripheral nervous system in zebrafish embryo	*ctgfa* *Fgf-dependent MAPK signaling* *Glucocorticoid signaling, Nrc31 receptor* *shh* *slit2* *slit3* *frizzeld-3a* *slit2* *frizzled-3a and wnt-8b unknown*	*Danio rerio* *Danio rerio* *Danio rerio* *Danio rerio* *Danio rerio* *Danio rerio*	Mokalled, et al (2016) Goldshmit, et al. (2012) Nelson, et al. (2019) Barresi, et al. (2005) Barresi, et al. (2005) Hofmeister, et al (2012). Hofmeister and Key, (2013). Lewis and Kucenas, (2014).
Axonogenesis response to injury	Axonogenesis Fibroblast-like cells during zebrafish spinal cord regeneration	*Wnt/β-catenin* *CollagenXIII*	*Danio rerio*	Wehner, et al. (2017).

3. Guidance cue molecular mechanisms in glial cell development of zebrafish forebrain

While glial development in zebrafish brain and spinal cord glia are different developmental processes, there is a common theme of extracellular guidance cue signaling that may provide further molecular and cellular insights for future studies in glial and neuronal regeneration. For instance, Shimizu and colleagues identified canonical Wnt/β-catenin signaling is required for radial glia differentiation and growth during physiologic and regeneration of radial glial cells after injury conditions [22]. We will first review known molecular mechanisms that govern glial cell development in the zebrafish forebrain (Figure 2B), and then provide future molecular studies that warrant investigations into zebrafish spinal cord regeneration. A glial bridge forms with a bipolar morphology in such a way that glial cells accumulate in a pattern directly across from each other to elongate across the lesion to promote axon regeneration [1]. In this context, it was vital to investigate what is guiding or communicating to the glial cells to accumulate in a bipolar nature and how that may play a role in spinal cord regeneration holistically. Glial bridging occurs during embryo development in the zebrafish forebrain and is guided by hedgehog regulated *slit* expression [23]. The bipolar morphology of glial cells to form bridges was found in regions lacking expression of two axon guidance molecules called *slit 1* and *slit 2*. In the context of glial bridge formation, it was demonstrated that Sonic Hedgehog signaling is required for glial bridging through regulating the expression of *slit 1*, *slit 2*, and *slit 3*. Inhibiting the function of *slit 2* and *slit 3* led to disruption to guiding glial cells to their desired location for glial bridging, and thus hindered the bipolar morphology and thereby halting axon guidance across the midline of the forebrain. On the other hand, inhibition of Slit1a led to reduced midline crossing, suggesting that *slit1a* plays a specific role in promoting midline crossing for axons.

The extracellular cues that control guidance molecule expression is an important feature that has important applications for future glial bridge formation. For instance, Wnt signals, such as Frizzled 3, has been implicated in axon crossing. Associated with guidance molecule *slit 2* to modulate midline axon crossing in the telencephalon is *Frizzled 3* (*Fzd-3*), a receptor required for the formation of the anterior commissure [24]. *Frizzled 3* is known to bind to the Wnt ligand family, a highly conserved extracellular domain rich in cysteine. In the telencephalon, Hofmeister and colleagues found that *Frizzled 3a* is required for commissural axon crossing and proper glial bridge patterning by modulating chemorepellent signal *slit2* expression. Hofmeister and colleagues found that knock down of *Frizzled-3a* results in a complete loss of the anterior commissure, which was then accompanied by loss of glial bridging and increase in *slit2* expression. The increase in *slit2* expression resulted in preventing commissural axon crossing along the midline of the telencephalon. Furthermore, the blocking of Slit2 activity post knock down *Frizzled-3a* rescued the anterior commissure which suggests that Frizzled-3a indirectly controls the growth of axons across the midline. Additionally, upon investigation of the Wnt genes, Wnt8b was found to genetically interact with Frizzled-3a to regulate axon guidance [25], loss-of- function mutation to either *Frizzled-3a* or *Wnt8b* resulted in increased *slit2* expression and thus hindrance to glial bridging. In addition to controlling expression of guidance molecules during zebrafish forebrain development, Wnt signals are also crucial in controlling expression of transcription factors, such as Frizzled-dependent control of Wnt canonical nuclear β-catenin target genes [25]. This interesting genetic interaction suggests that both Wnt and Frizzled work together to regulate expression of *slit2*. An important future path of investigation is whether Wnt or other signaling pathways, such as Sonic Hedgehog signaling, control the expression of neuronal guidance molecules like *slit2* in the context of glial bridge or axonal bridge formation after spinal cord regeneration.

Table 3. Future investigations into glial cell function during spinal cord injury.

Future Investigations into Glial Cell Function during Spinal Cord Injury
Develop optogenetic and cell ablation studies using genetic approaches to resolve the role of trailing astroglia function and glial cell bridge formation models across different model organisms.
Investigate the convergent and divergent molecular and cellular response of astroglial cells and glial bridge formation in response to extracellular signaling and severity of injury.
Investigate the role of termination signals that control glial cell bridge formation and its development for future translational therapies for spinal cord injuries.

4. Signal termination in glial bridge formation of the zebrafish spinal cord

We have reviewed the extracellular and intracellular signals that promote glial cell bridge formation after zebrafish spinal cord injury that include the following: ctgfa, Fgf signaling, and Wnt/β-catenin induced expression of CollagenXIII. One question that remains is how do additional extracellular and intracellular signaling act to inhibit glial cell bridging once completed? Termination signals provide the feedback inhibition to prevent glial cell formation in the zebrafish spinal cord. One of the terminating signals is the overexpression of Dkk1b, a secreted Wnt inhibitory protein (Figure 2A). Strand and colleagues demonstrated that β-catenin levels increase post injury, and both larval and adult zebrafish Wnt/β-catenin signaling is conserved. Interestingly, Dkk1b inhibits activation of the β-catenin reporter in the spinal cord, as well as disrupting locomotor recovery, glial bridge formation, and axon elongation. Together, these studies suggest a definite collaborative role for Wnt/β-catenin signaling in both adult and larval zebrafish [26]. In addition, a recent study has implicated glucocorticoid signaling through receptor Nr3c1 as a termination signal that inhibits glial bridge formation in adult zebrafish ependymal glia [27](Nelson, et al. 2019, Figure 2A). In summary, there are at least two signals—Dkk1 and Nr3c1—that act as signal termination for zebrafish glial bridge formation after spinal cord injury.

5. Discussion

The molecular mechanisms that underlie the novel ability to regenerate the spinal cord in zebrafish are necessary for developing possible therapies that may translate into human health. As mentioned in the Introduction, a network of extracellular and intracellular molecular positive and negative feedback device mechanisms acts to regulate cell growth, differentiation, and development (Figure 1). By extending lessons from *Drosophila* on "go and stop" signals in glial cell growth and differentiation [21], we propose that investigating the signals that promote and/or terminate each phase of the glial bridging in both central and peripheral nervous systems deserve future investigations. Furthermore, elucidating the mechanisms of glial cell heterogeneity, glial bridge cell specification and migration remain to be determined. There are many cellular factors that are involved in this mechanism and no one has investigated the transition points that stops one dimension of the mechanism and initiate the next. It is clear that specific growth factors, such as *ctgfa*, which is required for spinal cord regeneration, however it is not clear how such signals are terminated over space and time (Table 3). With advent of next generation single cell RNA-seq [28], optogenetics [29], and CRISPR-based cell lineage tracing methods [30], we envision the field to pursue the genetic compensation [31] and epigenetic compensation mechanisms involved at each transition point in glial bridge formation. Genetic compensation mechanisms warrant future investigations because the discovery of these will elucidate the genetic circuitry that control the molecular feedback cycle control (Figures 1 and 2). In light of the exciting research in glial biology and its role in disease and regeneration, we hope the scientific field will investigate the possible terminating factors that control glial cell bridging in the context of glial development and regeneration.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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