Review

Glial bridge ecology: cellular mechanisms that drive spinal cord regeneration in Zebrafish

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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. This unique aquatic species has been found to have the capacity to regenerate their spinal cord after injury. However, the complete molecular and cellular mechanisms behind glial bridge formation in the central and peripheral nervous systems upon glial cell injury remains unclear. This review paper focuses on the molecular mechanisms and cellular processes that underlie spinal cord regeneration in four initial phases: proliferation and initial migration; migration and differentiation; glial bridge formation; and remodeling. We propose that within these four phases the cellular mechanisms that underlie spinal cord regeneration each express a terminating signal that aborts one step of the process and initiates the next. Specifically, future studies would be devoted to investigate transmitting signals in the spinal cord injury micro-environment in hope to contribute to the understanding of underlying cellular mechanisms by connecting each process of spinal cord regeneration in zebrafish.

Keywords: glial bridge; *ctgfa*; Fgf signaling; MAPK signaling; *shh*; *slit2/3*; Wnt signaling; genetic compensation; glial bridge cycle; spinal cord regeneration; termination signal; central nervous system; peripheral nervous system; zebrafish.

1. Introduction

Zebrafish, unlike humans, have the capacity to regenerate their spinal cord after injury [1-3]. Glia are crucial in the context of development, disease progression, and injury response [4, 5]. In the context of spinal cord regeneration, the regenerative mechanism can be broken down into four initial phases following inflammation at the injury site: proliferation and initial migration, migration and differentiation, glial bridge formation, and remodeling [1]. Proliferation and initial migration is characterized in this context by the proliferative response by glial cells, which is an increase in numbers and initial move towards sit of spinal cord injury. The second phase, migration and differentiation, is defined by continued glial cell migration to the site of injury and by the glial cell bipolar morphology where "polar bases" of glial cells form across from each other at the lesion site [1]. Phase three is characterized by mature glial elongating over the lesion to form glial bridges. And to end the process is phase four: remodeling, characterized by cellular remodeling and excretion of cellular debris at the site of injury, which results in reconnection of central canal and axon growth (axonogenesis) across the lesion that in fully functional in nature. While there are recent review articles that focus on fruit fly *Drosophila* mechanisms and spinal cord regeneration mechanisms in mammals [6] , less is known regarding termination signals that control zebrafish glial bridge formation and remodeling 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 during each phase. By extending the "go and stop" signals in *Drosophila* and mouse model organisms in cell growth and differentiation [6] , we propose

a termination glial cycle repair model framework in which cells or its microenvironment provide a termination signal at each stage transition point during glial cell bridge formation and remodeling (Figure 1, Table 1).

2. From proliferation to initial migration: Generating the Pioneer Glial Bridge Cells

Phase one is characterized as the initiation step, in that this is the first process that is signaled to respond to injury to the spinal cord (Figure 1). To examine what is involved in this dimension of the regenerative mechanism as a whole, 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 acitivation is regulated by Fgf signaling and loss of function Fgf resulted in inhibition of glial bridges, disrupting the bipolar component of glial bridging. By hindering glial bridging this also hindered the regenerative groundwork for axonogenesis. A future research direction in how Fgf signaling is blocked to transition from initial migration of pioneer glial cells into forming differentiated glial bridge cells warrants future investigation.

2. Of Glial Cell migration and differentiation: A Guidance Molecule Twist to Glial Bridge Formation.

Phase two is defined by continued cellular migration and differentiation of cells in the context of accumulating to form a glial bridge (Figure 1). 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. 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 [7]. 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. By contrast, 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 [8]. *Frizzled 3* is known to bind to the Wnt ligand family, a highly conserved extracellular domain rich in cysteine. In the telencephalon Wnt signaling, through frizzled 3a, reduces expression of Slit2 in the midline of the forebrain, thus promoting glial bridge formation. 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 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 [9], a mutation or loss of function to either *Frizzled-3a* or *Wnt8b* resulted in increased slit2 expression and thus hindrance to glial bridging. To shed light into mechanisms of glial bridge

formation, the lessons of Wnt dependent axon guidance mechanisms illustrate a novel framework of how Wnt inhibitors, such as *Dkk1*, could be utilized to provide a transition point from cell growth and coordinated with its differentiated glial bridge cell state. Wnt signaling is believed to be linked in cellular migration and axon guidance during neural development, and upon reflection of the every phase described during spinal cord regeneration, we hypothesize that these same molecules interact in the regenerative mechanism in the spinal cord of zebrafish. Specifically, *slit 2* expression acts as a retardant for glial bridge formation, coupled with *frizzled-3a* and *wnt8b*, this could promote axonogenesis and thereby regeneration. However, it is essential to investigate transition points or cues that initiates one step in the regenerative mechanism to the next.

3. From Glial bridge formation to Remodeling: Integrating Molecular Cues and Signal Termination

This phase in the regenerative model is characterized by the maturing glia exhibiting long bipolar behavior which starts to fully elongate across the spinal cord to bridge the two sides of the damaged spinal cord. These elongated glia are consist of both proliferative and nondividing glia within the central canal. This formation of a bridge is dependent on molecular signals that control guidance molecule expression, such as Wnt receptor *frizzled*-dependent *slit 2* expression. This molecular readout defines phase 3 and the unique nature of zebrafish regenerative capabilities.

In addition to controlling expression of guidance molecules, Wnt signals are also crucial in controlling expression of transcription factors, such as Frizzled-dependent control of Wnt canonical nuclear Beta-catenin target genes [9]. This interesting genetic interaction suggests that the two (Wnt and Frizzled) work together to regulate expression of *slit2*. Furthermore, Beta-catenin levels increase post injury and we know that Wnt/B-catenin signaling in the injured spinal cord prevents axon elongation across the site of injury. Interestingly, Dkk1b, a secreted Wnt inhibitory protein, inhibits activation of the Beta-catenin reporter in the spinal cord, as well as disrupting locomotor recovery, glial bridge formation, and axon elongation. Together, this suggest a definite collaborative role for Wnt/B-catenin signaling in both adult and larval zebrafish [10]. The theme of genetic interactions and specific expression levels to mediate phases in the regenerative model seems to be patterned through expression a “terminating signal” that communicates to transition to the next step. For example, during *slit2* regulation, and accumulation of glial cells to begin building the glial bridge, there is obvious communication between regulatory guiding molecules and glia, thus there must also be a terminating factor that is expressed to initiate a transition to the next phase. Phase 3 is characterized by glial bridging, which influences axon elongation via activation of *Fgf*, which initiate phase of remodeling. Remodeling is the final step to the novel process, in which cellular debris and reorganizing occurs to promote absolute recover.

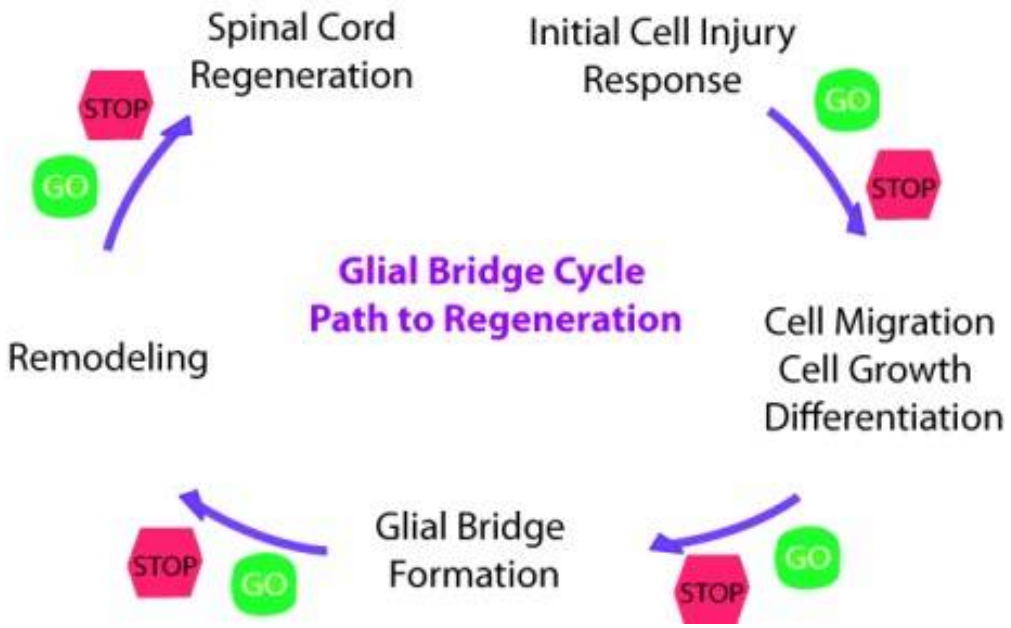

Figure 1. Glial Bridge Formation Cycle. (**A-C**) Upon glial cell injury, there are three phases: pre-glial bridge formation; glial bridge formation; and post-glial bridge formation. (**A**) Pre-glial bridge formation includes initial glial cell injury response, as well as cell growth/proliferation, glial cell bridge specification and differentiation and migration. (**B**) Glial bridge formation across different anatomical locations in either central nervous system (eg. forebrain, spinal cord) and peripheral nervous system. (**C**) Post-glial bridge formation include cell remodeling, including blood vessel repair and axon regeneration prior to tissue healing and regeneration.

Table 1. Highlighted Signaling Molecules Involved in Pre-Glial Bridge Formation, Glial Bridge Formation, and Post-Glial Bridge Formation during Spinal Cord Regeneration.

Phases of Glial Bridge Cycle	Cellular Process	Developmental System	Associated Signaling Molecules	Model Organism	References
Pre-Glial Bridge Formation	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); Kato, et al. (2011). [6, 11] Kucharova and Stallcup (2010, 2015) [12, 13]
	Cell Migration	To be identified and investigated	unknown	To be identified and investigated	
	Cell Growth and Homeostasis	Ventral nerve cord (VNC) Kon expressing cells	NFkB/Dorsal Kon-tiki Notch	*Drosophila*	Hidalgo and Logan (2017); Kato, et al. (2011); Losanda-Perez,

					et al. (2016). [6, 11, 14]
		NG2+ OPC cell growth	*NG2*	*Mus musculus*	Kucharova and Stallcup (2010, 2015) [12, 13]
	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	Pros	*Drosophila*	Hidalgo and Logan (2017); Kato, et al. (2011). [6, 11]
		NG2+ OPC cell growth	*NG2*	*Mus musculus*	Kucharova and Stallcup (2010, 2015) [12, 13]
Glial Bridge Formation	Glial Bridge formation post injury in spinal cord	Zebrafish Spinal Cord regeneration	*ctgfa* *Fgf-dependent MAPK signaling*	*Danio rerio*	Mokalled, et al (2016). [2] Goldshmit, et al. (2012). [1]
	Glial bridge formation in postopic commisure	Postoptic commissure (POC)	*shh*	*Danio rerio*	Barresi, et al. (2005). [7]
	Embryonic glial bridge formation in anterior commissure of zebrafish	Anterior Commisure (AC) optic nerve in central nervous system in zebrafish embryo	*slit2* *slit3* *frizzeld-3a* *slit2*	*Danio rerio* *Danio rerio*	Barresi, et al. (2005). [7] Hofmeister, et al (2012).[8]
	Glial bridge formation in forebrain commissural plate	Forebrain commissural plate	*frizzled-3a and wnt-8b*	*Danio rerio*	Hofmeister and Key, (2013).[9]
	Glial bridge formation in peripheral nervous system in zebrafish	Perineural glial bridge formation in peripheral nervous system in zebrafish	*Unknown* *Nkx2.2+ perineural glia*	*Danio rerio* *Mus musculus*	Lewis and Kucenas, (2014).[15] Clark, et al. (2014). [16]
Post-Glial Bridge Formation	Remodeling	Axon regeneration	*Fgf-dependent MAPK signaling*	*Danio rerio*	Goldshmit, et al. (2012)

4. 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. By extending lessons from *Drosophila* on "stop and go" signals in glial cell growth and differentiation [6], we propose that investigating the signals that promote and/or terminate each phase of the glial bridge cycle 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. With advent of next generation single cell RNA-seq [17], optogenetics [18], and CRISPR-based cell lineage tracing methods [19], we envision the field to pursue the genetic compensation [20] and epigenetic compensation mechanisms involved at each transition point in glial bridge formation. 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 may influence specific processes to stop and initiate the next process within the glial bridge cycle in both central and peripheral nervous systems.

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