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Spinal cord regeneration — the origins of progenitor cells for functional rebuilding

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Abstract

The spinal cord is one of the most important structures for all vertebrate animals as it connects almost all parts of the body to the brain. Injury to the mammalian spinal cord has devastating consequences, resulting in paralysis with little to no hope of recovery. In contrast, other vertebrate animals have been known for centuries to be capable of functionally regenerating large lesions in the spinal cord. Here, we will review the current knowledge of spinal cord regeneration and recent work in different proregenerative animals that has begun to shed light on the cellular and molecular mechanisms these animals use to direct cells to rebuild a complex, functional spinal cord.

Introduction

For centuries, functional spinal cord regeneration has fascinated scientists and propelled the field of regenerative research. Most mammalian species are unable to repair damage to the spinal cord after a traumatic injury, leading to the loss of motor, sensory, and autonomic function. In mammals, spinal cord injury results in a widespread apoptotic event, leading to the death of neurons and glial cells surrounding the injury site. This is followed by the formation of the glial scar, in which a compact barrier of reactive astrocytes, $NG2^{+}$ glia, and microglia surrounds the lesion site [1]. The formation of the glial scar, although intended to prevent further damage to surviving neurons, acts as a physical barrier that prevents axons from growing through the injury site and subsequently inhibits regenerative repair. Recently, the spiny mouse was shown to exhibit functional regenerative repair of its spinal cord after injury, which was attributed to the lack of glial-scar tissue surrounding the injury site [2••]. Similarly, a variety of other nonmammalian species lack the formation of glial-scar tissue after spinal cord injury and thus possess the intrinsic ability to regenerate their spinal cord (Table 1). Such regeneration-competent species include the zebrafish [3], lamprey [4], axolotl [5], and larval *Xenopus* [6]. Work in these model systems has been instrumental in identifying the conserved cellular mechanisms that promote successful repair of the central nervous system (CNS) and have demonstrated an important role for ependymal glia cells in

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spinal cord regeneration. In this review, we will discuss the role of ependymal glial cells in spinal cord regeneration, highlighting recent advances in understanding their origin and how these cells are activated after injury.

Ependymal cell response to injury

Ependymal glial cells are found across the entire CNS, lining the central canal of the spinal cord and the ventricles of the brain. In the spinal cord, the somas of ependymal glial cells form an epithelial barrier around the central canal and extend long radial processes containing end-feet-like structures to the pial surface (Figure 1a). Although ependymal glial cells are widely known for their important role in cerebral spinal fluid homeostasis, they have also been proven to play an important role in regenerative repair [7,8]. In regeneration-competent species, ependymal glial cells within the spinal cord express sex-determining region Y-box 2 (Sox2), a transcription factor that regulates stem cell pluripotency and self-renewal [9]. Even in adult organisms that are capable of regenerative repair, these ependymal glial cells are a relatively quiescent population of cells that rarely proliferate. After injury to the spinal cord, however, $Sox2^{+}$ ependymal cells behave as neural stem cells (NSCs) and rapidly proliferate to regenerate the missing portion of the spinal cord [10–13]. In the axolotl, CRISPR–Cas9-mediated deletion of Sox2 completely abolishes spinal cord regeneration [10]. Similarly, Sox2 knockdown using anti-sense morpholinos in *Xenopus* [14] and zebrafish [13] impairs NSC proliferation and spinal cord repair.

Early studies investigating ependymal cell responses to spinal cord injury identified a response zone within 500 μm of the injury site in which NSCs are activated and rapidly proliferate after tail amputation [15,16]. Following a more targeted transection injury, this response zone is found 500 μm rostral and 350 μm caudal to the injury site (Figure 1b) [17,18]. To better characterize the spatio-temporal dynamics of NSC activation within the response zone, more recent work in the axolotl utilized transgenic reporter animals to visualize cell-cycle dynamics *in vivo*. Using fluorescent ubiquitination-based cell-cycle indicator transgenic axolotls, ependymal cell activation was detected 800 μm from the injury site for the first 85 h after tail amputation. This recruitment zone gradually diminished, until the NSC border included a 450 μm response zone at 5 days post injury [19••]. Moreover, transcriptional profiling in *Xenopus* and zebrafish identified a significant enrichment for various cell-cycle regulators within the ependymal cell response zone 1 day after injury [20,21••]. Collectively, these studies have demonstrated that ependymal cells in the mature tissue directly adjacent to the injury site are mobilized to proliferate and regenerate the spinal cord and do so by accelerating their cell-cycle progression.

Activation of neural stem cells

In recent years, numerous studies have focused on identifying the injury-induced signaling pathways that activate NSC proliferation. In the axolotl, the microRNA, miR-200a, was shown to play an important role in regulating NSC activation. After spinal cord injury, mammalian glial cells upregulate a heterodimeric complex comprised of c-Fos and c-Jun to promote the expression of the glial fibrillar acidic protein (GFAP). This increase in GFAP expression results in reactive gliosis and inhibits neuronal regeneration in mammals

[22]. In the axolotl, miR-200a suppresses c-Jun expression in NSCs, ultimately promoting the formation of a noncanonical c-Fos/JunB heterodimer to prevent the upregulation of GFAP and promote regenerative repair. Further, miR-200a inhibition resulted in the c-Jun in axolotl ependymal glial cells, leading to a reduction in NSC proliferation [23]. In other species capable of CNS regeneration, including zebrafish [20], *Xenopus* [24], and lamprey [25], a similar upregulation of Fos and Jun expression is shown after injury. However, both *Xenopus* and lampreys lack a GFAP gene, indicating that the formation of a Fos/Jun heterodimer may regulate alternative glial-specific genes in these species [26].

To further identify the regulatory networks and genes that may activate NSCs, high-resolution profiling in *Xenopus* uncovered a markable increase in the Mechanistic target of rapamycinm (mTOR)-signaling pathway after spinal cord injury. Pharmalcogical inhibition of mTORC1 reduced the number of proliferating $Sox2^{+}$ NSCs, subsequently impairing spinal cord regeneration. This reduction in NSC proliferation was attributed to the inability of mTOR to activate genes involved in cell- cycle transition [21]. Numerous reports have identified mTOR as an important regulator of protein-translation initiation, which is a well-established process that underlies CNS regeneration [27]. Past work has largely investigated the role of mTOR in regulating regenerative repair of surviving neurons in the CNS after injury. In adult mice, virus-assisted conditional knockout of Phosphatase and Tensin Homolog (PTEN), a negative regulator of mTOR, resulted in mTOR over-expression and promoted robust retinal ganglion-cell (RGC) regeneration after injury [28]. Further work compared the intrinsic regenerative capabilities of regenerating sensory neurons to nonregenerating RGCs in the rat CNS. mTOR was highly upregulated and activated protein translation in regenerating sensory neurons. In contrast, nonregenerating RGC neurons exhibited a reduction in mTOR signaling and protein synthesis after injury [29]. Collectively, these studies have demonstrated the necessity of mTOR signaling in initiating protein synthesis in regenerating CNS neurons. However, more recent work in *Xenopus* may indicate that mTOR also plays an important role in NSC proliferation and will be an interesting pathway to examine in other regenerating systems.

Origin of neural stem cells

In the past two decades, particular emphasis has been placed on understanding ependymal cell dynamics after spinal cord injury. Through this work, we have gained a more thorough understanding of the cell-cycle dynamics and regulatory networks that activate ependymal glial cells after injury. However, the origin of NSCs remains somewhat unclear, with multiple reports, indicating that NSCs can arise through different mechanisms in regeneration-competent species. Whether these cells undergo dedifferentiation, transdifferentiation, or instead represent a developmentally derived progenitor-cell population remains unknown (Figure 2).

Dedifferentiation

Dedifferentiation has long been associated with regenerative repair, describing a mature cell that reverts into a progenitor/stem cell, giving rise to cells of its own lineage or potentially other cell lineages (Figure 2). A classic example of dedifferentiation was first inferred from

static images of a regenerating salamander limb, indicating that mature nucleated muscle fibers were pinching off single nuclei to form cells that would populate the injury site through a process of dedifferentiation [30]. It has been widely postulated for decades that many animals that can regenerate utilize dedifferentiation to form a mass of undifferentiated stem cells adjacent to the injury site termed a blastema, which will eventually differentiate to replace lost structures. The dedifferentiation of mature cells into pluripotent stem cells, called induced pluripotent stem cells (iPSc), has been shown *in vitro* utilizing mouse and human fibroblasts. After treatment with various factors, including Oct3/4, Sox2, c-Myc, and Klf4, mature fibroblasts reverted to a pluripotent stem cell state, where they were able to differentiate into multiple cell types (Figure 2) [31]. However, more recent work has indicated that dedifferentiation into pluripotent stem cells does not occur *in vivo* during regenerative repair. Classical work in the axolotl limb utilized genetic lineage-tracing tools to demonstrate that mature cells instead dedifferentiate into progenitors that retain lineage-specific cell markers, resulting in their differentiation into cells of a restricted lineage [32]. Similarly, the dedifferentiation of mature cells into progenitors of a restricted lineage plays a role in zebrafish heart and fin regeneration [33,34].

During spinal cord regeneration, dedifferentiation may also play an important role in successful regenerative repair. Interestingly, retrograde dextran tracing of axolotl neurons demonstrated that mature neurons in the spinal cord do not dedifferentiate after tail amputation. Instead, surviving neurons merely reincorporated axons into the regenerating spinal cord as early as 3 days post amputation [35]. In contrast, ependymal glial cells in the newt spinal cord were shown to upregulate various NSC markers after injury, thus indicating that these cells dedifferentiate into NSCs [36]. Consistent with these findings, gene expression profiling revealed a strikingly similar transcriptional landscape between regenerating axolotl NSCs and developing chick neuroepithelium stem cells, further suggesting that ependymal glial cells dedifferentiate into neural stem cells after injury [11]. In zebrafish, similar changes in the transcriptional profile of ependymal cells have been documented after a compression injury to the spinal cord. After injury, Foxj1a^{+} ependymal cells downregulate Foxj1a expression and rapidly proliferate. This reduction in Foxj1a expression in ependymal glial cells suggests that these cells dedifferentiate into a neural stem cell after injury [37]. Together, these studies indicate that the dedifferentiation of ependymal glial cells may be an important component to promote spinal cord regeneration after injury.

Transdifferentiation

Although cells in the regeneration blastema were originally thought to arise exclusively from the dedifferentiation of mature cells, further work identified the ability for mature cells to instead transdifferentiate and directly switch their cell lineage. Unlike dedifferentiation, trans-differentiating cells do not revert to a progenitor-cell state, but are instead converted directly into the required cell type of a different lineage for subsequent regenerative repair (Figure 2). Transdifferentiation of epithelial cells has become a well-established phenomenon during lens regeneration in urodele amphibians [38] and retina regeneration in *Xenopus* [39]. However, evidence of transdifferentiation has also been reported in urodele spinal cord regeneration.

Lineage-tracing experiments in the axolotl have indicated that ependymal glial cells transdifferentiate into cells of both an ectodermal and mesodermal lineage after tail amputation. Although ependymal cells often gave rise to spinal cord cells, they were also shown to migrate out of the spinal cord to dramatically switch their lineage and transdifferentiate into cartilage and muscle [40]. Interestingly, this phenomenon has not been reported in *Xenopus* spinal cord regeneration. Instead, mature spinal cord cells in the stump tissue exclusively give rise to newly regenerated spinal cord cells, and lack transdifferentiation potential [41]. However, *Xenopus* only regenerate their spinal cords as larval animals and lose this ability after metamorphosis. Thus, transdifferentiation may instead represent a process that is exclusive to animals that regenerate throughout life.

Embryonic origins

Salamanders appear to potentially use transdifferentiation [40] and dedifferentiation [36] to successfully regenerate the spinal cord. However, more recent reports have indicated that ependymal cells may in fact arise through different mechanisms that more closely represent developmental-like pathways. The multipotent potential of $Sox2^+$ NSCs to differentiate into neurons and muscle is reminiscent of a similar bipotent progenitor-cell population, neuromesodermal progenitors (NMps), that regulate axial elongation during development. NMps are classically defined as $Sox2^+$/$brachyury^+$ bipotent progenitor cells located in the tailbud of vertebrates that give rise to cells of an ectoderm and mesoderm lineage (Figure 2). Initially discovered in the mouse embryo [42], NMps have since been described in chick [43], quail [44], zebrafish [45], and humans [46]. In these developing embryos, NMps arise at the beginning of the primitive streak regression and persist through the remainder of axial elongation. However, it remains unclear at what specific timepoint NMps disappear from the tailbud, or if they persist into adulthood, albeit as a smaller population of progenitor cells that may contribute to maintenance and repair.

Multiple reports in the axolotl have demonstrated that ependymal glial cells can differentiate into cells of multiple lineages after tail amputation, including ectoderm and mesoderm [15,40]. Whereas after a more targeted spinal cord ablation injury, ependymal glial cells exclusively give rise to cells of an ectoderm cell lineage [17]. The ability for NSCs to exhibit mono- versus multipotent activity after different spinal cord injuries was recently shown to be regulated by the microRNA, miR-200a. In ependymal glial cells, miR-200a represses the mesoderm marker brachyury after an ablation injury to promote NSC differentiation into ectoderm. After tail amputation, miR-200a itself is downregulated in ependymal cells to promote the co-expression of NMp cell markers brachyury and sox2, enabling NSCs to give rise to either ectoderm or mesoderm [47••]. Further gene expression profiling of axolotl ependymal glial cells after tail amputation demonstrated that NSCs dramatically upregulate genes associated with NMp maintenance, including Cdx4 and the Wnt-signaling pathway [11]. These findings indicate that the cell state of ependymal glial cells is dramatically altered, depending on the injury context, and that NSCs appear to behave like NMps after tail amputation. Rather than representing the transdifferentiation of glial cells into ectoderm or mesoderm, tail amputation may instead represent the transition into a developmental-like progenitor-cell state. Whether similar events occur in other regeneration-competent species remains unclear. As NMps have been described in zebrafish embryos [45], it will be

interesting to investigate their persistence into adulthood in this regeneration-competent species, and their potential role in adult spinal cord regeneration.

Future perspectives

Although significant advances have been made in our understanding of ependymal glial-cell responses during spinal cord regeneration, many questions remain un-addressed. The origin and underlying factors that activate NSCs remain somewhat elusive, along with how these mechanisms may be conserved across species. Regenerative repair is a complex process and thus, is likely regulated by varying signaling pathways across species. In the past decade, the development of single-cell transcriptomics and genetic profiling has revolutionized scientific research, allowing researchers to specifically analyze entire transcriptomes of single-cell populations. As these more sophisticated single-cell RNA- profiling techniques become more widely accessible and feasible for regeneration-competent animals, future analyses of the ependymal cell profile after spinal cord injury will be instrumental in identifying the underlying factors that mediate NSC responses to injury. Moreover, many classical studies that identified trans-differentiating or dedifferentiating ependymal cells were performed over two-decades ago, when many transcriptomic techniques had not yet been established. It will be interesting to revisit these lineage-tracing experiments using single-cell RNA sequencing to identify different cell states (progenitor cell, mature differentiated cell) to further confirm whether ependymal cells undergo transdifferentiation or dedifferentiation. Comparing the transcriptional landscape of ependymal glial cells across species will also be important for understanding the conserved mechanisms and species specific pathways that exist to promote complex functional regeneration. In particular, determining whether specific signaling pathways necessary for CNS regeneration are conserved in the newly established spiny mouse spinal cord injury model will be an important step on the pathway toward promoting functional human spinal cord repair.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest.

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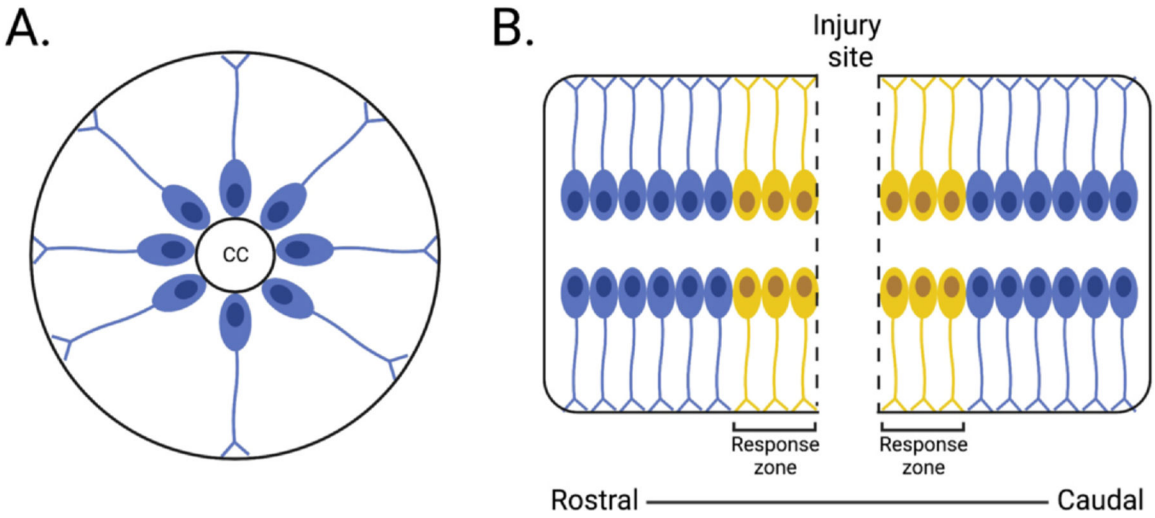

Figure 1.

Schematic diagrams of ependymal glial-cell organization in the spinal cord. **(a)** Cross-sectional view of the spinal cord, ependymal glial-cell somas line the central canal and extend radial processes toward the pial surface. **(b)** After injury to the spinal cord, ependymal glial cells ~500 μm rostral and ~350 μm caudal of the injury site are activated (response zone, yellow) and begin to rapidly proliferate, eventually differentiating into the necessary cell types required for regenerative repair.

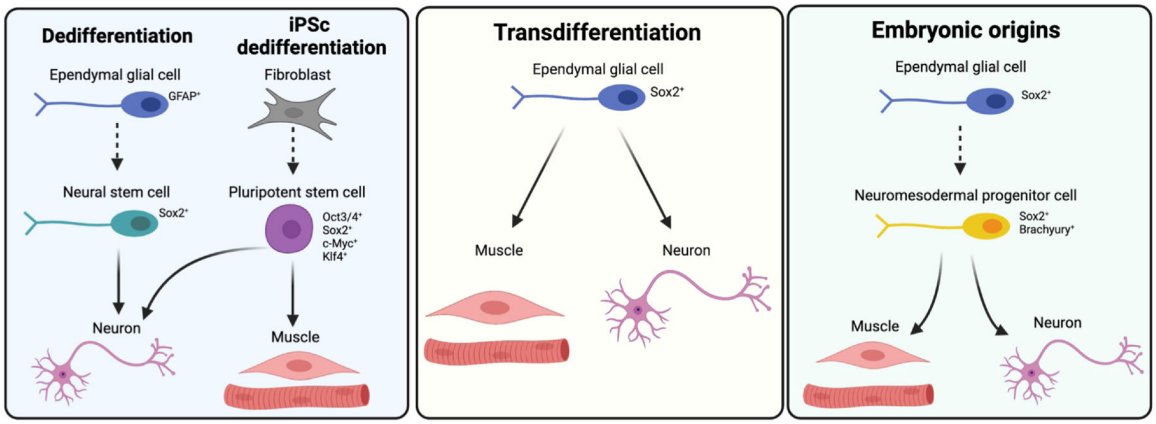

Figure 2.

Generation of stem cells. During regeneration of appendages in salamanders, several papers have shown data supporting the reversion of differentiated cell types such as muscle into multipotent progenitor cells that partake in regeneration. Similarly, in the spinal cord, ependymal glial cells can revert to a neural stem cell identity, this process is called dedifferentiation. The conversion of fibroblasts to iPSc originally by overexpression of specific genes is a process of dedifferentiation. In vivo cell-tracing experiments in axolotl have illustrated that cells in the spinal cord can form cells of other lineages, this is referred to as transdifferentiation. Interestingly, during development, a population of bipotent cells has been identified, which express the mesodermal marker Brachyury and the classical neural stem cell marker Sox2, these cells give rise to both ectoderm and mesoderm during development.

Table 1

List of species that form glial-scar tissue or that contain proliferating $Sox2^{+}$ neural stem cells after spinal cord injury.

	Human	Spiny mouse	Axolotl	Zebrafish	*Xenopus*	
					Larval	Adult
Glial-scar formation	Yes [1]	No [2]	No [21]	No [45]	No [46]	Yes [46]
Proliferating $Sox2^{+}$ NSCs in spinal cord	No	Unknown	Yes [47]	Yes [11]	Yes [46]	No [46]