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An ependymal cell quest

Identification and functional role of spinal cord

neural stem cells

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AN EPENDYMAL CELL QUEST

- IDENTIFICATION AND FUNCTIONAL ROLE OF SPINAL CORD NEURAL STEM CELLS

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ABSTRACT

Few injuries have as profound and long-lasting consequences as spinal cord injury. The primary areas of impaired function typically include sensation, mobility, bladder, bowel and sexual function. The economic, social and personal effects can also be devastating. Today there is no cure available but the discovery of neural stem cells in the adult spinal cord has raised the hope for a treatment. It has, however, proven difficult to identify these stem cells and several different cell types including ependymal cells, astrocytes and oligodendrocyte progenitors have been proposed to function as neural stem or progenitor cells in the adult spinal cord. We assessed the generation of new cells from these cell populations under physiological conditions and after spinal cord injury. In **Paper I,** we identify ependymal cells as latent neural stem cells. They have *in vitro* stem cell potential and are multipotent *in vivo* after injury, giving rise to scar-forming astrocytes and remyelinating oligodendrocytes. We also show that oligodendrocyte progenitors and astrocytes are lineage restricted progenitors in the adult spinal cord. We offer an integrated view of how several different endogenous cell populations generate new cells under physiological and pathological conditions.

Scar formation has traditionally been seen as an impediment to functional recovery after spinal cord injury. However, in **Paper II**, we show that scarring by ependymal cell-derived astrocytes is required to reinforce the tissue to prevent expansion of the lesion and further damage. We also identify ependymal cell progeny as a major source of neurotrophic support, and find substantial neuronal loss in the absence of this component of the scar tissue.

In order to modify the endogenous stem cell response following an injury it is important to understand if all spinal cord ependymal cells have stem cell properties, or if this feature is limited to a subpopulation. In **Paper III**, we show that ependymal cells are functionally heterogeneous with proliferating progenitors and quiescent stem cells. The latent neural stem cell population in the adult spinal cord is made up of a small subpopulation of ependymal cells that are activated, proliferate and give rise to a large number of cells both *in vitro* and in response to injury. This thesis is an ependymal cell quest that provides new insights to the identity and function of a latent neural stem cell population residing in the center of the spinal cord.

POPULAR SCIENCE ABSTRACT (SWEDISH)

I slutet av förra milleniet visade forskare för första gången att det bildas nya nervceller i vår vuxna hjärna och att dessa nervceller kommer från omogna stamceller. Sedan dess har jakten på hjärnans stamceller varit i full gång och förhoppningar har höjts om att kunna använda dem för att ersätta förlorade celler i en rad olika sjukdomar och skador i nervsystemet. Vid en ryggmärgsskada kapas nervfibrerna som leder signaler mellan hjärnan och resten av kroppen, vilket bland annat kan leda till förlamning. Efter en ryggmärgsskada aktiveras många olika sorters celler i ryggmärgen och där skadan skett bildas ett ärr. I det första delarbetet använder vi laboratoriemöss för att identifiera vilka av cellerna i ryggmärgen som är stamceller och hur de tillsammans med andra celltyper hjälps åt att bilda ärrvävnad. Ryggmärgens stamceller är så kallade ependymceller som täcker ytan kring de vätskefyllda hålrummen som finns i hjärnan och ryggmärgen. I detta delarbete visar vi även att stamcellerna är viktiga för läkningsprocessen, detta då de bildar stödjeceller som isolerar och skyddar överlevande nervbanor. Fram tills nu har forskare varit mycket oense kring huruvida ärret som bildas efter en skada är bra eller dåligt för läkningen. En av anledningarna till att det varit så omtvistat är att ärret består av flera olika celltyper och komponenter där stamcellerna bara utgör en viss del. I det andra delarbetet använder vi oss av ett genetisk trick för att stänga av stamcellerna i möss så att de inte kan aktiveras efter en skada. Vi visar att när ärrbildningen från stamceller blockerats växer sig skadan djupare med tiden och fler nervfibrer går av. Detta tyder på att stamcellerna hindrar skadan från att växa och bli större. Vi visar även att stamceller producerar livsviktiga överlevnadsfaktorer till nervceller i närheten av skadan utan vilka dessa nervceller dör. I det tredje delarbetet studerar vi ependymceller i mer detalj och visar att de består av flera olika grupper av celler varav endast en liten del är stamceller.

Huvudsyftet men den här avhandlingen är att identifiera ryggmärgens stamceller och visa att de är viktiga under läkningsprocessen efter en ryggmärgsskada genom att bilda både ärrvävnad som främjar läkning och stödjeceller som isolerar nervtrådar. I ryggmärgen finns det celler som har olika stamcellsegenskaper och om vi kan lära oss mer om hur olika sorters stamceller reagerar på skada så kan vi kanske styra dem till att bli bättre på att läka skador, potentiellt genom att bilda fler stödjeceller.

LIST OF PUBLICATIONS

I. Barnabé-Heider F, Göritz C, **Sabelström H**, Takebayashi H, Pfrieger FW, Meletis K, Frisén J. Origin of new glial cells in the intact and injured adult spinal cord. *Cell Stem Cell*, 2010, 7, 470-482.

II. **Sabelström H**, Stenudd M, Réu P, Dias DO, Elfineh M, Zdunek S, Damberg P, Göritz C, Frisén J. Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice. *Science*, 2013, 342, 637-40.

III. **Sabelström H**, Stenudd M, Ståhl F, Göritz C, Basak O, Clevers H, Brismar H, Barnabé-Heider F, Frisén J. Spinal cord ependymal cells are functionally heterogeneous with proliferating progenitor cells and quiescent stem cells. Manuscript.

Publications not included in this thesis:
Barnabé-Heider F, Meletis K, Eriksson M, Bergmann O, **Sabelström H**, Harvey M., Mikkers H, Frisén J. Genetic manipulation of adult mouse neurogenic niches by in vivo electroporation. *Nature Methods*. 2008, Feb;5(2):189-96.

Sabelström H, Stenudd M, Frisén J. Neural stem cells in the adult spinal cord. *Experimental Neurology*. 2013, doi: 10.1016/j.expneurol.2013.01.026

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LIST OF SELECTED ABBREVIATIONS

BDNF	Brain-derived neurotrophic factor
BLBP	Brain lipid-binding protein
BrdU	5-bromo-2´-deoxyuridine
CNTF	Ciliary neurotrophic factor
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalomyelitis
EdU	5-ethynyl-2´deoxyuridine
FoxJ1	Forkhead box protein J
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Glast	Glutamate aspartate transporter
HFH4	Hepatocyte nuclear factor/forkhead homologue 4
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor-1
NG2	Nerve/glial antigen 2
NGF	Nerve growth factor
PDGFRα	Platelet-derived growth factor receptor alpha
TGFβ1	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein

1 THE IDENTITY AND FUNCTIONAL ROLE OF SPINAL CORD NEURAL STEM CELLS

Stem cells are defined by two characteristic properties, the capacity to self-renew through mitotic cell division and the capacity to differentiate into specialized cell type(s) (*1, 2*). Stem cell properties are usually assessed on a tissue or cell population level, but in order to be classified as *a bona fide* or true stem cell both these characteristic must be present at the single cell level. Different stem cells are defined by their potential to self-renew and differentiate and this is often directly linked to the source of where you find them. The most potent stem cells are found in early embryos and as development progresses stem cells lose some of their original potential. In adult vertebrates, stem cells remain in most organs where they participate in tissue homeostasis and repair. Tissues that turnover fast have more pronounced stem cell activity, such as the skin or the intestine (*3*). Some adult stem cells divide frequently but the typical adult stem cell is quiescent and divides rarely to generate fast dividing progenitor cells (*4*). These progenitor cells are more restricted cells that are limited in their potential for differentiation and/or self-renewal (*5*). It is crucial to characterize differences and establish relationship between stem and progenitor cells in order to better understand tissue homeostasis, regeneration after injury and potential therapeutic applications.

1.1 ADULT NEURAL STEM AND PROGENITOR CELLS

1.1.1 Subventricular zone of the lateral ventricle wall

Neural stem cells can be found in the adult brain in a few distinct, well-defined stem cell niches, which are the microenvironment supporting and regulating stem cells. The first major region that produces new neurons in the adult mammalian brain is the subventricular zone lining the lateral wall of the lateral ventricles in the forebrain. Neurogenesis in the subventricular zone is important for certain aspects of the sense of smell as newly born immature neurons migrate from the subventricular zone, through the rostral migratory stream, to the olfactory bulb where they integrate as mature interneurons. Olfactory bulb neurogenesis has been implicated in olfactory memory

formation, odorant discrimination and social interactions (*6*). The neurogenic niche in the subventricular zone consists of several different cell types.

The neural stem cells share ultrastructural and antigenic features with astrocytes and are often referred to as type B cells (*4, 7*). Some type B cells are mitotically active whereas others are quiescent stem cells (*4, 7, 8*). Activated type B cells undergo cell division to self-renew and generate transit-amplifying progenitor cells (TAPs or type C cells) that will generate large numbers of immature neurons, namely neuroblasts, which will migrate in long chains through the rostral migratory stream to the olfactory bulb where they will integrate as functional interneurons. Type B cells can also generate cells of the oligodendrocyte lineage (*9*).

The lateral ventricle walls are lined by post-mitotic ciliated ependymal cells, which function as a border between the cerebrospinal fluid and the subventricular zone. The neurogenic niche also contains blood vessels, which are closely associated with type B and type C cells (*10, 11*). Proliferating cells in the subventricular zone are often associated with blood vessels or the extracellular matrix around them (*11, 12*), suggesting that factors derived from the vasculature may regulate both neural stem and progenitor cells and thus establishing an important role of the niche.

1.1.2   Dentate gyrus of the hippocampus

Another major region that produces new neurons in the adult mammalian brain is the dentate gyrus of the hippocampus (*2, 13-15*). New neurons in the adult dentate gyrus have been implicated in learning and memory (*16-19*), and alterations in neurogenesis in this brain region has been linked to psychiatric diseases such as depression (*20, 21*) as well as to neuroinflammation (*22*) and epilepsy (*23*). Interestingly, anti-depressant drugs and increased physical activity have shown an increased proliferation of cells in the subgranular zone of dentate gyrus (*20, 21*). The neurogenic niche in the subgranular zone has been studied to some extent in rodents and new hippocampal neurons are born in the subgranular zone of the dentate gyrus. Radial astrocytes function as neural stem cells in the subgranular zone, similar to observations in development and in the adult subventricular zone. These radial astrocytes give rise to intermediate progenitor cells, referred to as type D cells (*24*) or type II progenitors (*25, 26*), that proliferate

and gradually mature into functional neurons (*24, 25, 27*). Nonradial, or horizontal, astrocytes have also been proposed to act as primary progenitor cells in the adult subgranular zone (*28*). Nonradial astrocytes lack radial processes and some have extensions parallel to the dentate granule cell layer (*28*). The stem cell potential of nonradial astrocytes and their lineage relationship to other stem and progenitor cells remain unclear (*29, 30*).

Several studies have investigated neurogenesis in the human brain but this has proven to be difficult due to limitations of the methods used. Studies in rodents have relied on administration of thymidine analogues, such as BrdU, to label dividing cells and at a later time point analyzing labeled presumably newly born neurons. Since BrdU is incorporated into the genomic DNA it is not given to humans. However, in a study from the late '90's several terminal cancer patients were given BrdU as a potential treatment. The brains of these patients were later donated to research and constituted a milestone as it showed newly born neurons in the hippocampus indicating neurogenesis in adult humans (*31*).

More recently, the Jonas Frisén lab has devised a new method to study cell turnover in the human brain using retrospective birth dating of cells by measuring the levels of carbon-14 in the genomic DNA (*32*). This is possible due to the above-ground nuclear bomb tests during the Cold War that made the atmospheric levels of carbon-14 spike. Several recent studies have been able to confirm that there is no neurogenesis in the adult human cerebral cortex, but that new neurons are integrated into the hippocampus of adult humans, with comparable neuronal turnover rates in middle-aged humans and mice (*31-34*). Surprisingly, although adult humans generate neuroblasts in the subventricular zone, there is no detectable neurogenesis in the adult olfactory bulb (*35-37*) instead there is integration of newly born interneuron in the striatum, which is adjacent to the subventricular (*38*). This highlights the importance of confirming animal studies in humans, as some biological processes can vary greatly or even be profoundly different.

1.1.3 The third ventricle by the hypothalamus

A third, less studied neurogenic region in adult brain is the third ventricle by the hypothalamus. An increasing number of studies in rodents are suggesting that tanycytes in the ependymal layer that line the third ventricle have neural stem cell properties and generate both neurons and glia under physiological conditions, as well as multipotent neurospheres *in vitro* (*39-44*). The hypothalamus is involved in functions regulating feeding, sleep, reproduction, circadian rhythms, stress response, blood pressure, and core body temperature (*45*). Neurogenesis in the third ventricle has been suggested to be involved in the control of the body's energy balance (*46-48*).

Tanycytes are a type of ependymal cell, which are ciliated cells lining the entire ventricular system of the adult brain. Hypothalamic tanycytes have elongated radial glial-like morphology with long basal processes, similar to neural stem cells in other regions of the developing and adult brain. A stem cell niche-like architecture, similar to the one in the subventricular zone, also exists in the hypothalamus region lining the third ventricle (*49*). However, this neurogenic niche needs to be studied in greater detail in order to assess the regulation and neural stem/progenitor cell potential of hypothalamic tanycytes, as well as, the functional role of hypothalamic neurogenesis. In addition, the potential involvement of hypothalamic tanycytes in adult human neurogenesis needs to be addressed.

1.2 ADULT SPINAL CORD NEURAL STEM AND PROGENITOR CELLS

Because of the lack of specific markers and challenges associated with *in vivo* studies, *in vitro* stem cell assays have been remarkably useful. Indeed, although no neurogenesis occurs in the adult spinal cord, *in vitro* assays have shown that stem cells reside along the entire axis of the central nervous system (*50-52*). One of the most common ways to define a neural stem cell is by the neurosphere assay (*50*) where putative neural stem cells are propagated *in vitro* in the presence of growth factors to produce self-renewing free-floating clusters of cells, called neurospheres, which can be induced to differentiate into multiple neural lineages upon growth factor withdrawal. There are only few distinct neurogenic areas in the adult brain, however neurospheres can be isolated from the entire neuroaxis of the adult central nervous

system (*50-52*). Astrocytes rarely proliferate outside the neurogenic regions in the adult brain, but after injury they can start proliferating and even acquire stem cells properties *in vitro* (*53-57*). Oligodendrocytes are constantly being generated throughout the adult brain by lineage restricted progenitors (*54, 58*) and some studies have suggested that these cells could acquire stem/progenitor cell properties *in vitro* (*58, 59*). Ependymal cells, which are mostly quiescent in the adult brain, have been suggested to form neurospheres in some regions (*44, 50*). Studies using BrdU incorporation showed that several glial cell populations (including astrocytes, oligodendrocytes and ependymal cells) were mitotically active in the adult spinal cord (*60*), leaving the door open to the identity of spinal cord neural stem cells.

1.2.1 Astrocytes are not stem cells in the adult spinal cord

Astrocytes are highly active cells in the adult central nervous system where they have complex functions that play critical role in homeostasis, including interactions with synapses and regulation of blood flow (*61, 62*). In addition, subtypes of astrocytes are *bona fide* neural stem cells in the brain. There is no neurogenesis in the adult spinal cord but there are proliferating glial cells (*60*) and astrocytes represent one of these proliferating populations (Paper I). To assess the stem- and progenitor cell potential of astrocytes in the adult spinal cord we used the inducible Cre-LoxP system to generate a transgenic mouse (Figure 1). The promoter of an astrocyte-specific gene was used to drive the expression of a tamoxifen-dependent Cre recombinase, called CreER (*63*). This mouse was then crossed with a reporter strain that upon Cre-mediated recombination expresses a reporter gene, for example YFP (*64*), allowing inducible and permanent labeling of astrocytes and their progeny. In Paper I, we utilized Connexin30 (Cx30) which is a gap junction protein expressed in astrocytes in the central nervous system (*65-67*). The Cx30-CreER mouse line allows specific recombination of astrocytes in the adult brain (*68*) and spinal cord (Paper I). More than 70% of Sox9-positve astrocytes in the adult spinal cord were recombined in the Cx30-CreER mouse (Paper I). These cells also expressed the astrocytic markers GFAP and S100β, but not the oligodendrocyte lineage markers Sox10, NG2 or PDGFRα (Paper I). Based on morphology and marker expression these astrocytes were divided into three subpopulations, one in the white matter expressing Sox9 and GFAP and two different ones in the grey matter. A small number of recombined cells in the grey matter were

A

Transgenic mouse lines

Ependymal cells: FoxJ1-CreER

Resident astrocytes: Cx30-CreER

Oligodendrocyte progenitors:

Olig2-CreER

B

FoxJ1 CreER

R26R STOP YFP
loxP loxP
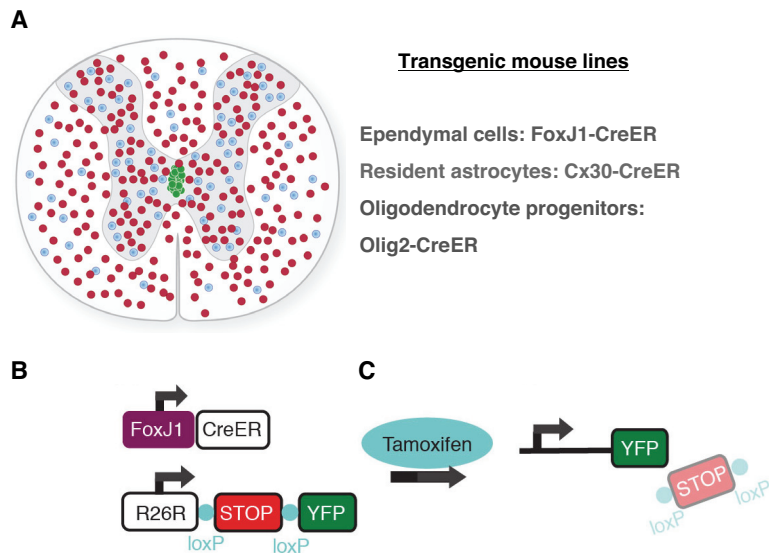
C

Tamoxifen

YFP
STOP loxP
loxP

Figure 1. Fate mapping of cells in the adult spinal cord with the inducible Cre-LoxP system.

A. To specifically label different cell populations in the adult mouse spinal cord, the tamoxifen-dependent Cre-recombinase (CreER) was expressed under different promoters. The human FoxJ1 promoter was used to specifically label ependymal cells (green), a BAC containing the Connexin30 (Cx30) promoter for resident astrocytes (red), and a knock-in of CreER into the endogenous Olig2 locus was used to label oligodendrocyte progenitors (blue).

B-C. The cell-type specific promoters drive the expression of CreER and upon tamoxifen administration CreER gets relocalized from the cytoplasm into the nucleus where is recombines LoxP sites in a reporter allele (R26R) to remove a Stop codon and allow reporter gene expression (YFP). This allows an inducible and permanent YFP labeling of the different cell populations, which is inherited to their progeny. *Adapted from Paper 1 and Paper II.*

located close to the ependymal layer with a bipolar morphology and expressing Sox9 and GFAP. The vast majority of recombined astrocytes in the grey matter have the elaborate morphology of protoplasmatic astrocytes and express Sox9, lower levels of GFAP mostly around the cell body and surprisingly, the oligodendrocyte lineage marker Olig2. Since these cells did not express any other oligodendrocyte lineage marker, they were considered to be astrocytes (Paper I). This has also been reported in the brain for Olig2 expression in astrocytes (*54*). Cx30-CreER astrocytes did not produce progeny of other lineages in the uninjured spinal cord and their number stayed constant over time (comparing 5 days and 4 months after recombination; Paper I), suggesting that Cx30-CreER astrocytes are limited progenitor cells that proliferate to compensate for some cell loss to maintain the size of their cell populations. Ge and

colleagues reported similar findings in the postnatal cortex where differentiated astrocytes only generated new astrocytes (*69*).

To assess the *in vitro* stem cell potential of astrocytes in the adult spinal cord, we generated neurospheres from Cx30-CreER mice. We found one recombined neurosphere in all the primary cell cultures (n=3 mice, average 93 neurospheres per culture; paper I). We collected and passaged that one neurosphere to assess its stem cells potential since restricted progenitor cells have been shown to give rise to primary neurospheres with limited self-renewal and/or differentiation potential (*70, 71*). The single recombined Cx30-CreER-derived neurosphere did not generate a secondary neurosphere demonstrating that is was generated from a restricted progenitor cell (Paper I). While astrocytes harbor neural stem cell properties in neurogenic niches of the brain, they do not contribute to more than their own population in the spinal cord during physiological conditions.

1.2.2 NG2 cells are restricted oligodendrocyte progenitors

Oligodendrocytes are a numerous glial cell type in the adult central nervous system and they are scattered throughout the white and grey matter of the brain and spinal cord. Oligodendrocytes insulate axons with myelin sheaths to allow fast saltatory conduction and protect nerve fibers that travel long distances throughout the brain and spinal cord (*72*). Mature oligodendrocytes are post-mitotic but can be replenished from progenitor cells (*73*). Oligodendrocyte progenitors are also found in white and grey matter throughout the central nervous system and they express the markers PDGFRα, Olig2, NG2 and the oligodendrocyte lineage marker Sox10 (*74*). Oligodendrocyte progenitors, also referred to as NG2-positive cells or polydendrocytes, represent the main proliferating cell population in the intact spinal (Paper I) (*75*) and have been proposed as neural stem cells in the adult spinal cord, however, recently it has been highly debated whether oligodendrocyte progenitors are committed oligodendrocyte lineage cells or if they can generate other neural cell types (*74, 76*).

Several studies using different approaches to identify and fate map oligodendrocyte progenitor cells have attempted to assess their stem cell potential *in vivo*. This has, however, proven to be difficult with the use of traditional tracing methods as they have

innate limitations. For instance, studies using viral labeling of proliferating cells have led to the suggestion that oligodendrocyte progenitors in the adult spinal cord have neural stem cell properties and produce cells of multiple lineages *in vivo* (*75, 77-79*). However, lentivirus injection to label cells can be unspecific which makes it difficult to identify the founder cell and, in addition, the virus and injection itself causes an injury, which could affect the outcome (*75, 77, 80*). Fate mapping in transgenic mice with a promoter-driven expression of a reporter gene, such as LacZ or GFP, is dependent on the expression of the promoter by potential oligodendrocyte progenitor cell progeny, otherwise the label is transient and lost as cells differentiate, in addition, there is also the risk of false positives if another lineage turn on the promoter (*81, 82*). It was not until the development of the Cre-loxP transgenic mouse system that fate mapping and lineage relationship between oligodendrocyte progenitor cells and their progeny could be determined. Some initial studies using the Cre-loxP system to fate map potential oligodendrocyte progenitors from embryonic stages suggested that they have greater lineage potential and generate protoplasmic grey matter astrocytes in addition to oligodendrocytes (*78, 83*). One concern with the Cre-LoxP system is that cells will recombine and express the reporter gene (e.g. GFP or LacZ) as soon as the Cre-driving reporter is active. This often happens during development and thus one needs to carefully characterize which cells are recombined in the adult since this may not reflect promoter activity and/or the expression of the endogenous gene.

In order to determine the stem cell potential of oligodendrocyte progenitor cells, we used the tamoxifen inducible Cre-LoxP system (Figure 1). We used the Olig2-CreER mouse to label oligodendrocyte progenitors. Olig2 is expressed in oligodendrocyte progenitors and mature oligodendrocytes in the adult spinal cord (*75, 84*) and it is an important transcription factor in oligodendrocyte development and it is also required for the specification of oligodendrocyte progenitor cells (*85, 86*). Both mature oligodendrocytes and oligodendrocyte progenitors are recombined in the Olig2-CreER mice during development (*86, 87*) and in the adult spinal cod (Paper I). Recombined Olig2-CreER cells are scattered within white and grey matter in the adult spinal cord and almost all recombined Olig2-CreER cells express Olig2 and Sox10 and a quarter of them are mature APC-positive oligodendrocytes (Paper I). The recombination efficiency is rather low within the oligodendrocyte lineage (>5% of all Sox10-positive oligodendrocyte lineage cell; Paper I) but it is more efficient in oligodendrocyte progenitors (approximately 40% of NG2-positive cells; Paper I). A

very small fraction (Approximately 1%) of recombined Olig2-CreER cells is Olig2-positive grey matter astrocytes expressing Sox9 and GFAP, which corresponds to <1% of grey matter astrocytes. The very inefficient recombination in Olig2-positive astrocytes allowed us to distinguish between astrocytes and oligodendrocyte lineages cells in Olig2-CreER and Cx30-CreER mice (Paper I). Since some protoplasmic astrocytes appears to be positive for the oligodendrocyte lineage marker Olig2 (Paper I) (*54*) one should be careful to distinguish between cell types based on single markers, and use several lineage markers in combination with morphology to characterize, for instance, oligodendrocyte lineage cells.

We were able to confirm that oligodendrocyte lineage cells are indeed the main proliferating cell population in the intact spinal cord with 80% of BrdU-positive cells (Paper I). To identify the progeny of these proliferating cells we analyzed the phenotype and numbers of recombined Olig2-CreER cells after tamoxifen-induced recombination. The total number of recombined cells increased significantly 4 months after recombination due to a large increase of recombined mature oligodendrocytes (Paper I). The number of recombined progenitor cells stayed constant indicating that these cells both self-renew and produce new mature oligodendrocytes (Paper I). Previous work has suggested that adult spinal cord oligodendrocyte progenitors are bipotent and generate astrocytes (*75, 77-79*), but our genetic lineage analysis does not support this. Our results rather indicate that spinal cord oligodendrocyte progenitors are similar to those in the forebrain, which have been demonstrated by similar genetic fate mapping strategies to give rise to new oligodendrocytes but not astrocytes (*54, 58*). Our findings that oligodendrocyte progenitors are restricted oligodendrocyte lineage progenitor cells in the adult spinal cord have been confirmed by other research groups using the PDGFRα-CreER and NG2-CreER mouse lines (*88, 89*).

To assess the *in vitro* stem cell potential of oligodendrocyte progenitors in the adult spinal cord, we generated neurospheres from Olig2-CreER mice but we could not find any recombined neurospheres demonstrating a lack of *in vitro* stem/progenitor cell properties (Figure 2D; Paper I). Other studies have also failed to generate multipotent neurospheres from oligodendrocyte progenitors, however it seems possible to generate fate-restricted neurospheres under certain injury conditions (*59*). Thus, oligodendrocyte progenitors are, in spite of their extensive self-renewal capacity *in vivo*, better defined as restricted progenitor cells than stem cells.

1.2.3 Ependymal cells are neural stem cells in the adult spinal cord

Ependymal cells are cells with motile cilia lining the ventricular walls in the brain and the central canal in the spinal cord. Several functions have been suggested for ependymal cells, including propulsion of cerebrospinal fluid (CSF), and a barrier function between CSF and brain parenchyma (*90*). Ependymal cells are thought to filter CNS molecules to protect the brain and spinal cord from harmful substances in the CSF, optimize the dispersion of neural messengers in the CSF and move cellular debris in the direction of bulk CSF flow (*90*). Ependymal cells constitute a heterogeneous group of cells, with variations in both morphology and marker expression (*91-96*). The two most abundant ependymal cell morphologies in the spinal cord are multi-ciliated cuboidal cells and single ciliated tanycytes (*94, 96, 97*). Tanycytes have a basal process, which can contact blood vessels or neurons suggesting interactions between ependymal cells and the surrounding niche (*94, 96*). Tanycytes in the third ventricle have been reported to have neuroendocrine functions with long processes going into the hypothalamus parenchyma (*98*). A third, less common, ependymal cell is the radial ependymal cell, which reside in the dorsal and ventral poles of the central canal and extend long processes towards the pial surface (*94*).

In contrast to mammals, postnatal spinal cord neurogenesis occurs in several non-mammalian model organisms, including adult fish and urodele amphibians (*99, 100*). These species are also able to regenerate their spinal cord throughout their lifespan. Ependymal cells act as neural stem cells and they show morphological similarities to radial glia cells, which are the neural stem cells during development of the central nervous system (*99, 101*). Radial glia cells give rise to ependymal cells in mammals and some ependymal cells retain the expression of the radial glial markers RC1 and BLBP (*90, 94, 95, 102*). Several other molecular markers associated with immature neural cells are expressed in the ependymal layer, including the intermediate filaments Vimentin and Nestin, and the neural stem cell associated markers CD15, Musashi1, CD133/prominin-1, Sox2, Sox3 and Sox9 (*93-95*). Tanycytes in the third ventricle of adult rodent brains represent a specialized type of ependymal cells that share many morphological characteristics and marker expression with radial glial cells and they have been shown to have stem/progenitor cell properties, as mentioned above (*39, 43, 44*). Ependymal cells in other regions of the adult brain are post-mitotic (*4, 103-105*). However, ependymal cells in the uninjured adult spinal cord

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have been shown to proliferate (*51, 60, 94*). The proportion of BrdU-labeled ependymal cells is similar to that of oligodendrocyte lineage cells (4%–5%) but substantially lower than for astrocytes (<1%) (Paper I). However, oligodendrocyte progenitors proliferate approximately 10 times more frequently than ependymal cells making the oligodendrocyte lineage the dominating proliferative cell population during physiological conditions (Paper I). Ependymal cells do not generate progeny of any other cell fate under physiological conditions suggesting that they only proliferate to maintain their population (*94*) (Paper I).

The Jonas Frisén lab has been trying to identify cells with *in vitro* neural stem cell potential in the adult spinal cord for a long time and early studies indicated that neurosphere-forming cells are situated close to the central canal (*51*). This study had some methodical limitations as injection of fluorescent dyes into the cerebrospinal fluid to fate map ependymal cells may not be as specific as later developed methods and the dye gets diluted over time as cells divide (*51*). Some subsequent studies by other groups were either able to verify the findings using microdissection of the spinal cord (*106*) or find sphere-forming cells in medial and lateral parts of the spinal cord (*59, 84*). In a more recent study, the Frisén lab was for the first time able to genetically fate map ependymal cells and showed that only ependymal cell-derived neurospheres are multipotent and can be expanded for several passages (*94*). Pfenninger and colleagues verified that the *in vitro* neural stem cells potential of the adult spinal cord is confined to the ependymal cell population by flow cytometric isolation (*105*).

Genetic fate mapping of ependymal cells is possible using the FoxJ1 promoter to drive CreER expression in the adult mouse spinal cord (Figure I) (*94*). FoxJ1 (also called HFH4) is expressed in cells with motile cilia or flagella (*107*), which is specific for ependymal cells lining the central canal of the adult spinal cord (*94*). We have assessed the *in vitro* neural stem cells potential of three distinct cell populations in the adult spinal cord and only recombined FoxJ1-CreER ependymal cell-derived neurospheres are self-renewing and multipotent (Figure 3D; Paper I).
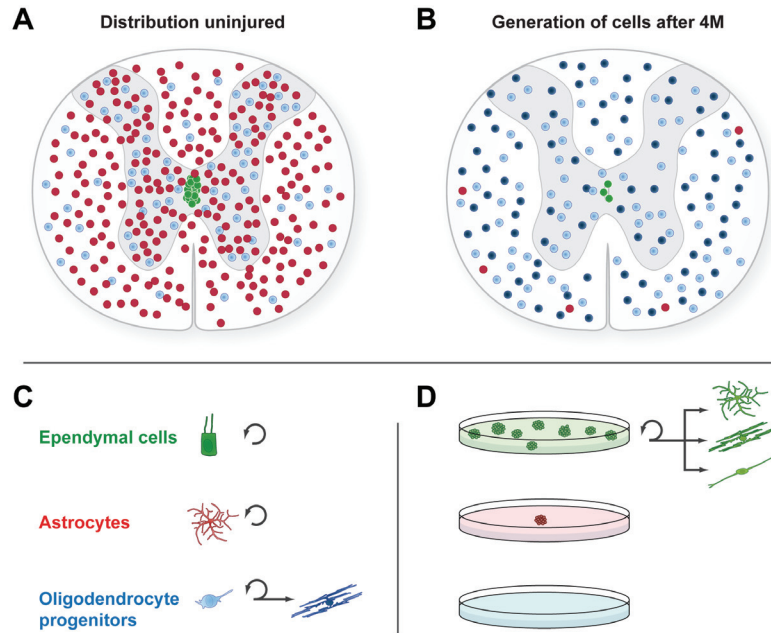
Figure 2. Distribution of new cells in the adult spinal cord.
(A) Distribution of ependymal cells (green), resident astrocytes (red) and oligodendrocyte progenitors (blue).
(B) Generation of new cells 4 months after recombination. Cells are depicted at 50% of their numbers in (A) and (B).
(C) Ependymal cells and resident astrocytes self-renew, whereas oligodendrocyte progenitors both self-renew and generate mature oligodendrocytes.
(D) Ependymal cells generate multipotent self-renewing neurospheres *in vitro*. Resident astrocytes generate few primary neurospheres that cannot be passaged further.
Based on data from Paper I. *Figure from (108).*

Recombined Olig2-CreER oligodendrocyte progenitors did not generate any neurospheres and the one Cx30-CreER astrocyte-derived neurosphere could not be passaged (Figure 3D; Paper I). Thus, we confirmed that ependymal cells are the *in vitro* neural stem cells of the adult spinal cord (*94, 105*) (Paper I). We also found that ependymal cell and astrocyte proliferation is restricted to self-duplication to maintain their populations in the uninjured spinal cord, whereas oligodendrocyte progenitors self-renew and give rise to an increasing number of mature oligodendrocytes (Figure 3C). Therefore, virtually all *in vitro* stem cell potential is restricted to one class of glial cells in the adult spinal cord, namely ependymal cells.

1.3   SPINAL CORD INJURY

Spinal cord injury typically results in a loss of motor function and sensory input below the level of the lesion. There is a significant loss of neurons and glial cell, and a dramatic increase in proliferation followed by recruitment of cells to the injury site (*51, 80, 109, 110*). Various types of cells from the spinal cord and the blood are recruited such as glial cells, pericytes, leukocytes, microglia and thrombocytes (*111*), which contribute to the scar that is formed at the lesion site. The scar is composed of a fibrotic core surrounded by a glial scar. The glial scar can serve as a major barrier for regenerating axons, but it also helps to restrict the inflammation to the lesion epicenter and protect the intact tissue from further damage (*112-115*). The glial scar is generated by astrocytes derived from resident astrocytes and ependymal cells (Paper I) (*94, 116*), and the fibrotic scar tissue is formed by perivascular cells, including type-A pericytes (*109, 117, 118*). When oligodendrocytes are lost, axons are left partly demyelinated and unable to efficiently propagate impulses. The loss of oligodendrocytes is thought to cause further impairment of neurological function in partial spinal cord injuries, the most common type of injury in humans, which is why rapid remyelination is important (*119, 120*). Both oligodendrocyte progenitors and ependymal cells have previously been demonstrated to generate myelinating oligodendrocytes after spinal cord injury (*54, 58, 94*). Neurons that are lost after injury are not replaced since there is no neurogenesis following spinal cord injury (*80, 82, 121, 122*).

Most studies on cell fate mapping within the injured spinal cord have focused on following the progeny of one specific population of dividing cells. To get an integrated view of how different neural cell populations contribute to new cells after spinal cord injury, we characterized the distribution and phenotypes of progeny from Olig2-CreER mice for oligodendrocyte progenitors, Cx30-CreER mice for resident astrocytes, and FoxJ1-CreER mice for ependymal cells at different time points after a dorsal funiculus incision. The cut was made transversely without reaching the grey matter or central canal and extended rostrally with microsurgical scissors to span one segment (*94*). All three cell populations increased their proliferation after injury and about 80% of all recombined cells were labeled with BrdU 5 days after injury (Paper I). The number of recombined cells increased 5.5 fold in the ependymal FoxJ1-CreER line and about 2-fold in the astrocytic Cx30-CreER and oligodendrocytic Olig2-

CreER lines by 2 weeks after injury. The total numbers of recombined cells were then maintained at almost the same level for up to 4 months. This shows that the recruited endogenous cells persistently contribute to new cells at the lesion site. Despite the dominance in proliferation of oligodendrocyte progenitors under physiological conditions (Figure 2B), they come in third place for the net cell contribution 4 months after spinal cord injury (Figure 3B; Paper I). This is due not to a reduced production of progeny by Olig2-CreER oligodendrocyte lineage cells, which is doubled, but to a larger increase in the production of progeny by FoxJ1-CreER ependymal cells (39% of new glial cells) and Cx30-CreER astrocytes (34% of new glial cells) (Figure 3B; Paper I). The careful comparison of the injury response between the different mouse lines reveled that ependymal cells, resident astrocytes and oligodendrocyte progenitors produce progeny that occupy complimentary domains after spinal cord injury. Under physiological conditions ependymal cells are exclusively found surrounding the central canal, however, after injury ependymal cell proliferation increases and the progeny is found at the center of the lesion as scar-forming astrocytes and in small numbers scattered in the parenchyma as oligodendrocytes (Figure 3A; Paper I) (*51, 80, 82, 94, 121, 123*). Cx30-CreER astrocyte progeny accumulates in the perimeter of the glial scar, outside the fibrotic core and the ependymal-derived astrocytic scar. In chronic lesions ependymal cells and resident astrocytes are each contributing to about half of the glial scar. This is consistent with previous reports showing an ependymal cell and/or astrocytic origin of the glial scar (*51, 82, 94*). All Cx30-CreER recombined cells maintained astrocytic features, expressing Sox9 and high levels of GFAP. The phenotype of grey matter astrocytes was altered after injury, with few expressing Olig2 and now displaying a less ramified and more elongated morphology (Paper I).

Following demyelinating injuries such as focal demyelination and experimental autoimmune encephalomyelitis (EAE), oligodendrocyte progenitors were shown to generate Schwann cells, which are the myelinating cells in the peripheral nervous system, as well as a small number of astrocytes (*124, 125*). To assess the differentiation potential of oligodendrocyte progenitors after spinal cord injury, we analyzed the phenotype of recombined cells in Olig2-CreER mice. We found that oligodendrocyte progenitor progeny is increased throughout the entire injured segment and consist exclusively of oligodendrocyte lineage cells, 97% of new oligodendrocytes are generated from Olig2-CreER recombined cells, compared to 3%

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A Distribution of new cells after injury (4M)

B Contribution to new cells

C

Ependymal cells

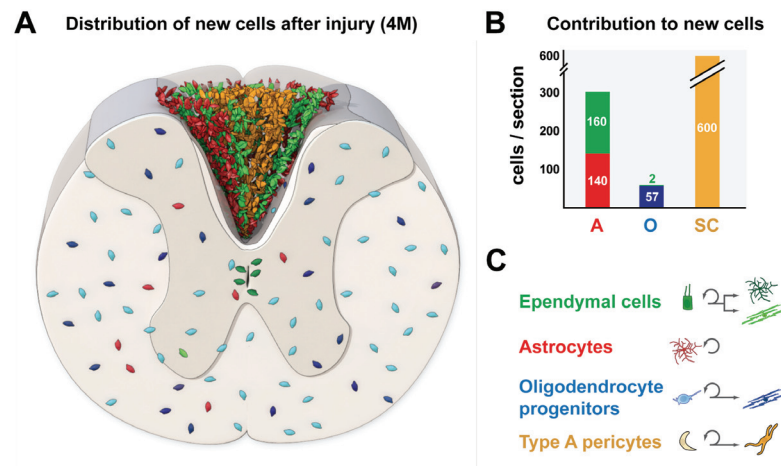Astrocytes

Oligodendrocyte progenitors

Type A pericytes

Figure 3. Distribution of new cells 4 months after spinal cord injury.
(A) Ependymal cells (green), resident astrocytes (red), oligodendrocyte progenitors (blue) and type-A pericytes (orange) contribute to new cells in complimentary domains after injury.
(B) Origin of astrocytes (A), oligodendrocytes (O) and stromal cells (SC) from the different cell lineages indicated by different colors in C.
(C) Ependymal cells self-renew and give rise to scar-forming astrocytes and oligodendrocytes after injury. Resident astrocytes self-renew and oligodendrocyte progenitors self-renew and give rise to mature oligodendrocytes, whereas type-A pericytes self-renew and generate stromal cells after injury.
Based on data from Paper I and from (*109*). *Figure from (108).*

from FoxJ1-CreER recombined ependymal cells (Figure 3B; Paper I). These findings demonstrate that resident astrocytes and oligodendrocyte progenitors are lineage-restricted progenitor cells whereas ependymal cells are multipotent after traumatic spinal cord injury.

Following spinal cord injury proliferation is increased *in situ* and the number of *in vitro* neural stem cells is also increased and a larger number of faster growing neurospheres can be isolated after an injury (*75, 84, 126, 127*). Cortical astrocytes are similar to spinal cord astrocytes in that they do not generate cells of other fates during physiological or injured conditions (*53, 69*) (Paper I). However, cortical astrocytes can give rise to neurospheres after injury (*53, 56, 57*). To investigate if spinal cord astrocytes also gain *in vitro* stem cell properties after injury, we generated neurospheres from Cx30-CreER mice. Similar to physiological conditions, only one neurosphere was generated from Cx30-CreER astrocytes (n= 3 mice, average 298 neurospheres per culture; paper I) and this neurosphere could not be further passaged.

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Thus, there is considerable heterogeneity with regard to stem cell properties between astrocytes in different parts of the adult central nervous system. We continued to assess the neurosphere-forming potential of ependymal cells and oligodendrocyte progenitors after injury and we were able to confirm that self-renewing, multipotent neurospheres are exclusively generated from recombined FoxJ1-CreER ependymal cells (Paper I).

In paper I, we provided an integrated view of how different cell populations contribute to new cells during homeostasis and after injury. We defined the final distribution of the progeny, with ependymal-derived astrocytes mostly within the lesion, astrocyte-derived astrocytes forming the lesion borders, and oligodendrocyte progenitor-derived and, to a lesser extent, ependymal-derived new oligodendrocytes present in spared tissue surrounding the lesion. We showed that ependymal cells are latent neural stem cells in the spinal cord and their neurosphere-forming potential is enhanced after injury, whereas astrocytes and oligodendrocyte progenitors are lineage restricted progenitors devoid *of in vitro* stem cell potential.

1.4 REGENERATIVE APPROACHES

1.4.1 Cellular replacement by transplantation

Spinal cord injury results in the loss of several different cell types, such as neurons and oligodendrocytes. The rapid initial loss of cells is increased over time due to secondary damage. Therefore, many studies have focused on cellular replacement by transplantation of different kinds of stem cells or stem cell-derived cells to promote functional recovery after spinal cord injury (*128*). The spinal cord environment is highly gliogenic and not permissive for neurogenesis. Ependymal cells with the potential to differentiate into neurons *in vitro*, do not contribute to any detectable neurogenesis in the injured spinal cord and only generate astrocytes and oligodendrocytes (*94*) (Paper I). Similar observations have been reported when adult or embryonic neural stem cells are transplanted into an injured spinal cord as they also only give rise to astrocytes and oligodendrocytes (*129-133*). Interestingly, when such neural stem cells are transplanted into the neurogenic environment of the hippocampus, they do make new neurons (*132*). Thus, the environment in the adult spinal cord appears to be highly restrictive for neuronal differentiation and/or fail to support

neuronal survival. This was further confirmed by Hofstetter and colleagues by showing that neural stem cells engineered to express the pro-neural gene Neurogenin2 (Ngn2) almost exclusively differentiate to neurons *in vitro*, but only to glial cells upon transplantation into the injured spinal cord (*133*). The increased generation of oligodendrocytes improved functional outcome compared to transplantation of unaltered neural stem cells (*133*). Several other studies have shown that increasing the number of oligodendrocytes by transplanting oligodendrocyte progenitor cells and embryonic or neural stem cells directed to an oligodendrocyte fate can improve functional recovery (*134-136*). Given the gliogenic nature of the adult spinal cord, it might seem more feasible to promote functional recovery by modulating or enhancing the generation of non-neuronal cells than by trying to replace lost neurons. However, a recent study by Lu and colleagues, suggested that the non-permissive inhibitory environment in the adult spinal cord could be overruled if fetal neural stem cell transplants are embedded in fibrin matrices containing a cocktail of growth factors including BDNF, IGF-1, HGF, EGF and bFGF (*137*). The authors reported an increased neuronal differentiation, axonal growth and connectivity in the grafts and improved functional recovery (*137*). Although the long-distance growth of the grafted cells in this study is impressive, it could be possible that some of the beneficial effects are from the administration of growth factors in the fibrin matrices.

1.4.2 Modulating the injury microenvironment

In cell transplantation studies it can be difficult to elucidate where the beneficial effects come from. Cellular replacement has its obvious benefits as it can replace lost cells and cell grafts can help bridging the scar allowing axonal regeneration. Increasing levels of growth or survival factors can reduce cell death and promote a more permissive environment. Transplantation of non-neural cells has also proven to be beneficial for functional recovery after injury. This is believed not to be due to cellular replacement but rather that transplanted cells modulate the microenvironment in beneficial ways. For instance, it is highly controversial if transplanted mesenchymal stem cells can generate neural progeny while there is more evidence supporting that their beneficial effect is through modulation of the injured spinal cord environment (*138, 139*). Mesenchymal stem cells synthesize a number of neurotrophic cytokines that stimulate axonal growth and neuronal survival, including BDNF, NGF, and VEGF (*139-141*).

Transplantation of fibroblasts modified to produce neurotrophic factors or administration of the growth factors alone can improve recovery (*136, 142-149*). Several mechanisms are behind the beneficial effects of growth factor treatments and some of the proposed mechanisms include increased sprouting, axonal growth, neuronal and/or oligodendrocyte survival (*136, 144, 150, 151*). In addition, growth factor treatments could potentially modulate the endogenous neural stem cell response to an injury, such as by increasing the proliferation of ependymal cells (*106, 146, 152*). A recent study by Hawryluk and colleagues showed that *in vitro* neurosphere cultures isolated from the adult rat spinal cord express numerous growth factors such as CNTF and TGFβ1 (*153*). We have previously shown that ependymal cells are the neurosphere-forming cells in the spinal cord (Paper I) (*94*). The finding that ependymal-derived cells can produce neurotrophic factors *in vitro* is very interesting from a therapeutic perspective since ependymal cells play a central role in scar formation and could potentially constitute an endogenous source of neurotrophic support after injury (Paper I) (*82, 94*).

1.4.3 Modulating the injury response from endogenous cells

It has been shown that promoting oligodendrogenesis can improve functional recovery after spinal cord injury (*75, 133-136*). Both oligodendrocyte progenitors and ependymal cells generate new oligodendrocytes after injury (Paper I) (*94, 154*). From a clinical perspective it would be ideal to be able to stimulate the production of oligodendrocytes from these endogenous stem/progenitor cells to avoid invasive cell transplantation therapy and the need for immunosuppression. However, it is not clear if manipulating ependymal cells towards an oligodendrocyte fate, at the expense of scar-forming astrocytes, will indeed lead to a more beneficial outcome. The function of the glial scar has long been under discussion and several studies have demonstrated that it has both positive and negative effects on recovery (*112, 114, 155-157*). The classical view of the scar as only bad is now becoming more challenged as more and more studies are showing clear positive effects. One of the reasons why the scar harbors distinct, almost opposite, functions could be that it is made up by several different cell types, which could have detrimental or beneficial effect on recovery (Paper I) (*108*). The traditional view of the scar is that it is a physical barrier preventing axons to grow through the scar which limits regeneration (*156, 157*). Reactive astrocytes in the glial

scar express a number of inhibitory factors, such as chondroitin sulfate proteoglycans and several mouse studies have shown beneficial effects from blocking reactive gliosis by knocking out GFAP and Vimentin, which are highly expressed in reactive astrocytes (*158-160*). However, GFAP and Vimentin are expressed by both reactive resident astrocytes and by a subset of ependymal cell progeny. Therefore, it is difficult to know exactly how the scar is affected in these animals before we know if the two different astrocytic populations have similar or separate functions (Paper I and Paper II). In addition to studies demonstrating detrimental effects of the glial scar, there are many that, on the contrary, report various beneficial effects. Specifically, Faulkner and colleagues investigated the role of reactive astrocytes after spinal cord injury using a transgenic mouse that express herpes simplex virus-derived thymidine kinase under the control of the GFAP promoter (GFAP-TK mouse) to kill dividing astrocytes upon administration of the antiviral drug ganciclovir (*161*). They found that elimination of reactive astrocytes resulted in a massive infiltration of inflammatory cells, a larger lesion volume, an increased loss of neurons and a worsened functional outcome (*112*). One potential caveat using this method is that it leads to the death of dividing cells, which in itself can exacerbate the damage. A recent study by Bardehle and colleagues showed that perivascular astrocytes in the cortex divide after injury. If the same cells exist in the spinal cord it could also help explain some of the detrimental effects seen in the GFAP-TK mouse such as the increased infiltration of immune cells (*162*).

More recently, other studies have used the Cre-LoxP system to conditionally knock out genes involved in reactive gliosis specifically in Nestin or GFAP expressing cells. The gene of interest is flanked by two loxP sites, which upon Cre-mediated recombination will excise the gene sequence between them to create a knock out (Figure 4). Nestin is expressed by both ependymal cell-derived and astrocyte-derived reactive astrocytes and specific deletion of signal transducer and activator of transcription 3 (Stat3), which is a key player in astrocyte differentiation (*163*) in either Nestin or GFAP expressing cells resulted in disrupted glial scar formation after injury (*113-115*). Using these transgenic mice, it was confirmed that elimination of reactive astrocytes results in a massive infiltration of inflammatory cells and worsened functional outcome (*113-115*). Okada and colleagues also showed that deletion of the protein suppressor of cytokine signaling 3 (Socs3) in Nestin-positive cells increased the reactive state of astrocytes, leading to the opposite effect: increased astrocyte migration, premature glial scar formation and improved recovery (*114*). Taken

together, these studies suggest that the glial scar prevents inflammatory processes from spreading to the healthy tissue around the lesion.

In summary, the glial scar has been shown to have clear beneficial and detrimental effects on functional recovery after a spinal cord injury. It is tempting to speculate that the two different astrocyte populations derived from ependymal cells and resident astrocytes could be differentially contributed by these different properties of the glial scar. It is crucial to further understand the function of the different glial scar components to be able to modulate the injury response in order to improve recovery.

1.4.4 Resident neural stem cells restrict tissue damage and neural loss after spinal cord injury

To study the role of ependymal cell progeny after spinal cord injury, we used transgenic mice to specifically ablate proliferation of ependymal cells and thereby block the generation of ependymal cell progeny after injury (Figure 4). This transgenic line was made by crossing the ependymal cell-specific FoxJ1-CreER mouse with an inducible knockout of Ras (denoted rasless) (Figure 4; Paper II) (*94, 164*). There are 3 Ras genes, H, N and K, which are required for cells to go through G1 phase to mitosis and the 3 genes are partially redundant (*109, 164*). The rasless mouse is homozygous for H-Ras and N-Ras null alleles and homozygous for floxed K-Ras alleles (Figure 4). Mice with a double knockout of two Ras genes will appear normal but upon recombination, when the third Ras gene is excised, cells are no longer able to proliferate (*164*). Thus, the FoxJ1-rasless mouse (i.e. FoxJ1-CreER:R26R-YFP:H-Ras -/-:N-Ras -/-:K-Ras fl/fl) allows for both specific and inducible ablation of ependymal cell proliferation and expression of YFP in ependymal cells in the adult spinal cord after tamoxifen administration. We used mice with the same genotype as controls, but they received oil rather than tamoxifen to avoid recombination.
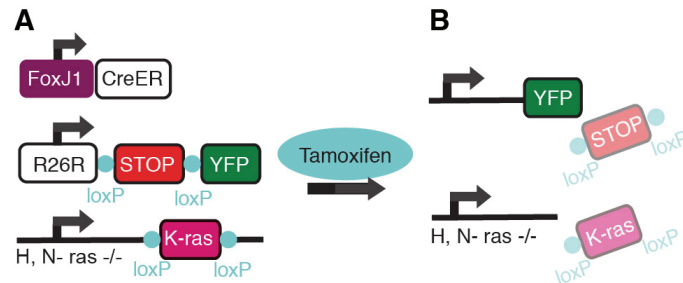
Figure 4. Ablating the generation of ependymal cell progeny in FoxJ1-rasless mice.

A-B Schematic outline of the strategy to block proliferation of ependymal cells. An ependymal cell-specific inducible CreER-loxP mouse line (FoxJ1-CreER:R26R-YFP, described in Figure 1) was crossed to a conditional Ras knockout mouse. The mouse is homozygous for H-ras and N-ras null alleles and K-ras is flanked by two loxP sites that upon Cre-mediated recombination will excise the K-ras gene to get a rasless mouse. *Adapted from Paper II.*

To characterize the impact of the impaired ependymal cell response we studied chronic injuries, 14 weeks after a dorsal incision injury. We found that 79% of FoxJ1-rasless mice, the mice without ependymal cell response, displayed scars with various degrees of tissue defects, ranging from minimal gapping at the injury site to single large cysts (Paper II). Cyst formation is common after spinal cord injuries in for instance humans and rats, but not in mice (*165*), and our control mice never developed a cyst. All control mice had dense glial scars at the site of the lesion (Paper II). This suggests that ependymal cell progeny is needed to restrict the spreading of the lesion. Indeed, further analysis of the lesion site revealed that the injuries were deeper, there was less spared tissue and the spinal cords were thinner, implying atrophy of the tissue in FoxJ1-rasless mice compared to control mice (Paper II). Since analysis and surgeries were made blindly, we assumed that the injuries were initially comparable between the groups. This suggests that the injuries grew deeper in a secondary process after the initial cut.

To assess the consequence of enlarged lesions, we examined the integrity of the corticospinal tract, which is a major axonal tract located immediately ventral to the lesion in this paradigm. We found that the corticospinal tract was spared in most control mice, whereas it was disrupted in the majority FoxJ1-rasless mice (1 of 14 and 9 of 14 corticospinal tracts were disrupted in control and FoxJ1-rasless mice, respectively; Paper II). This suggests that scarring by ependymal cells progeny is

required to reinforce the tissue to prevent secondary enlargement of the lesion and severance of axons spared by the initial injury (Figure 5). To assess the dynamics of this process, an additional cohort of mice was followed over several weeks with longitudinal magnetic resonance imaging (MRI). The lesions in control mice were reduced with 17% of their initial depth 9 weeks after injury, whereas the lesions in FoxJ1-rasless mice had gradually grown larger by 29% of their original depth and some animals had developed a single large cyst (Paper II). This shows that ependymal cell progeny is important to restrict an injury and without them the injury will keep growing (Figure 5). The longitudinal MRI also allowed us to confirm that FoxJ1-rasless mice developed a significant atrophy of the spinal cord over time (Paper II). The observed atrophy raises the question of what kind of tissue is lost in the absence of ependymal cell progeny. We found a 20% increase in neuronal loss in FoxJ1-rasless mice in segments adjacent to chronic lesions (Paper II). Already at 2 weeks after injury, an increased number of cleaved caspase 3-positive apoptotic neurons were found in animals without ependymal cell progeny (Paper II).

Neurotrophic factor expression is typically upregulated in response to spinal cord injury (*145, 153, 166*). We assessed the expression levels of several neurotrophic factors and found significant attenuation of the up-regulation of CNTF, HGF, IGF-1 and TGFβ1 mRNA in FoxJ1-rasless mice 2 weeks after injury (by 47-77% compared to injured control mice; Paper II). Immunohistochemistry in the injured spinal cord of control mice showed that CNTF, HGF and IGF-1 are synthesized by ependymal cell-derived astrocytes (Paper II). Administration of these neurotrophic factors has been shown to improve functional recovery and/or rescue neurons after spinal cord injury (*144, 145, 167*). These results suggest that ependymal cell progeny play a role in keeping neurons alive after injury, possibly through supplying neurotrophic factors in the spinal cord.

To verify that the phenotype was specific for the absence of ependymal cell progeny and not a general consequence of impaired scarring, we looked in another mouse line with impaired scarring. Göritz and colleagues have shown that Glast-rasless mice, with blocked type-A pericyte response, failed to seal the lesion and regain tissue integrity (*109*). We were, however, not able to find any other similar phenotypes. The Glast-rasless mice did not have lesions extending deeper or increased atrophy compared with control animals and, in addition, they did not have increased neuronal loss suggesting that these are FoxJ1-rasless specific phenotypes (Paper III).
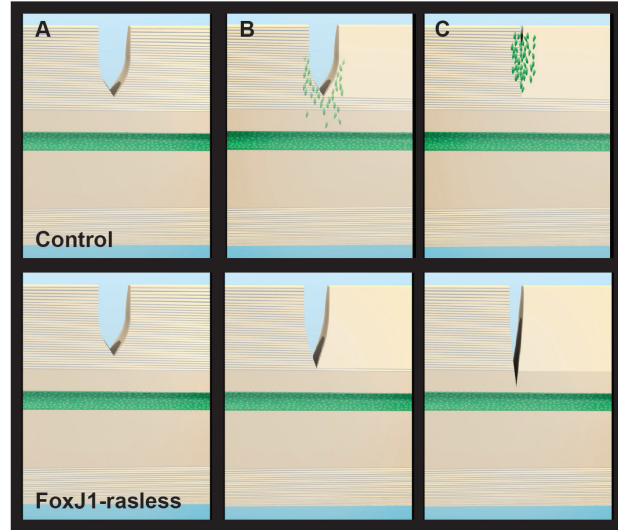
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Figure 5. Ependymal cell progeny restrict tissue damage after spinal cord injury.

A-C. The illustration shows a simplified view of lesion development after spinal cord injuries in mice, with (Control) and without (FoxJ1-rasless) the ependymal cell injury response. Ependymal cells (green) in control mice (upper panel) respond to injury by producing progeny that migrate to the lesion site (upper panel in B) where they contribute to the forming glial scar (upper panel in C). Without ependymal cell progeny (lover panel), the injury gradually expands (lower panel in B and C) and more nerve fibers are cut off caudal to the lesion (right in illustration). Illustrations from Mattias Karlén.

Thus, we have proposed two novel functions for ependymal cells, the latent neural stem cells of the adult spinal cord. The first is a scaffolding function that prevents the injury from growing after the initial damage to restrict secondary injury. The second function is in supplying neurotrophic support that keeps neurons from dying in the toxic environment after spinal cord injury. In conclusion, glial scars have been proposed to have both beneficial and detrimental effects on recovery. We have identified the origin of a beneficial part of the glial scar, which could be an attractive target to modulate in response to injury.

1.5   NEURAL STEM CELL HETEROGENEITY

Several studies have described heterogeneity of neural stem and progenitor cells in the brain. Below follows some major examples of functional, regional and morphological heterogeneity within neural stem cell niches.

1.5.1   Heterogeneity in the subventricular zone

The subventricular zone of the lateral ventricle wall is one of the most studied neurogenic regions in brain. Stem/progenitor cells in the subventricular zone are generating various kinds of neural cells, including astrocytes, oligodendrocytes and several different kinds of olfactory bulb interneurons (*4, 7, 9*). GFAP-expressing stem cells (type B1 cells) can generate both oligodendrocyte progenitors and transit amplifying (type C) cells (*168*). These committed progenitors can divert from one lineage to another when their oligodendrogenic or neurogenic fate determinants are lost (*169*), suggesting that they have a common bi-potent neural stem cell (type B cell) that can give rise to both oligodendrocyte lineage and neuronal lineage cells (*170*). On the contrary, viral targeting or genetic lineage tracing studies have shown that neural stem cells are heterogeneous when it comes to generating different neuronal subtypes in the olfactory bulb. It has been shown that neural stem cells display functional regional diversity and specific subtypes of interneurons are made within specific regions along the dorso-ventral and rostro-caudal axis of the adult subventricular zone (*30, 171-177*). Merkle and colleagues showed that the regional specification of neural stem cells is cell-intrinsic and maintained even after transplantation or after multiple passages in culture (*174*).

The progenitor cell state of type C cells can be reverted to a stem cell state by exposure to growth factors as shown by their ability to generate self-renewing, multipotent neurospheres *in vitro* (*178*). Thus, certain stimuli can induce dedifferentiation of cells. Astrocytes outside the neurogenic niches in the brain do not actively divide (*53, 54, 58*). However, astrocytes can become reactive and start proliferating after injury. Buffo and colleagues were able to show that a subset of reactive astrocytes in the cerebral cortex gain *in vitro* stem cell properties after a stab wound injury and were able to generate multipotent self-renewing neurospheres (*53*). Another study, by Carlén and colleagues showed that another post-mitotic,

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differentiated glial cell type gain multi-lineage potential after injury. Ependymal cells in the neurogenic niche by the lateral ventricle wall can give rise to both astrocytes and neuroblasts after a stroke (*103*). However, unlike spinal cord ependymal cells, these forebrain ependymal cells do not self-renew and become depleted in the process of generating progeny (*103*). Oligodendrocyte progenitors are also recruited after a brain injury, but they are restricted to their own lineage and do not show any *in vitro* stem cell potential (*54, 88*). Thus, it seems that an injury can induce various stem/progenitor cell responses in local quiescent glial cells in the adult brain.

1.5.2 Heterogeneity in the dentate gyrus

The neural stem and progenitor cells of the adult hippocampus consist of a heterogeneous pool of cells that display different marker expression (*15, 27, 179-182*) and morphologies (*24, 28*). There has been conflicting evidence about whether hippocampal neural stem/progenitor cells are self-renewing or depleted during the process of generating new neurons (*29, 30*). Differences in differentiation potential have also been reported for hippocampal stem/progenitor cells. These apparently contradicting results might reflect the coexistence of several cell populations with different stem cell properties. Indeed, a recent study by Decarolis and colleagues suggests functional heterogeneity of hippocampal radial glia-like stem cells using two different transgenic mouse lines (Glast-CreER and Nestin-CreER) to fate map potential stem/progenitor cells (*179*). They found that both cell populations contribute to neurogenesis, however, the Glast-CreER cell population seemed to be much smaller and was suggested to represent dormant or "long-term" stem cells, whereas the Nestin-CreER cell population was fast dividing and suggested to represent "short-term" stem cells. When neurogenesis was manipulated, either by stimulation or ablation of cells, they found that Glast-CreER radial glial cell contributed to neurogenesis whereas Nestin-CreER radial glial cells did not (*179*). This heterogeneity could indicate that there are different subpopulations of stem cell-like cells in the hippocampus raging from unipotent committed progenitors to self-renewing multipotent neural stem cells. Further studies need to investigate the relationship between different heterogeneous cell populations and mechanisms governing their stem cell potential.

1.5.3 Heterogeneity within the ependymal layer

Ependymal cells are present throughout the entire axis of the adult central nervous system and they display regional differences. Ependymal cells in the lateral ventricle wall are post-mitotic, whereas they have stem cell properties in other regions such as the third ventricle and the spinal cord (*4, 43, 90, 94*). Although ependymal cells in different regions share many features they still constitute a heterogeneous group of cells (*91-96*). Pfenninger and colleagues showed that there are differences in gene expression between ependymal cells in the lateral ventricle wall and the spinal cord (*105*). Spinal cord ependymal cells show higher expression of genes involved in cell division, cell cycle regulation and chromatin stability and they share some transcripts with radial glia cells of the embryonic forebrain such as Fen1, which is important for telomerase activity (*105*). This goes well with their physiological base-line proliferation and latent stem cell potential. Post-mitotic ependymal cells in the lateral ventricle wall express genes regulated by TGFβ family members. These genes are involved in neuronal specification and keeping cells in undifferentiated states and could potentially help explain how forebrain ependymal cells can turn into neuroblasts after stroke (*103, 105*).

In addition to gene expression, the ependymal cells are also morphologically heterogeneous. For instance, the ependymal layer in the spinal cord consist of equal numbers of cuboidal ependymal cells and tanycytes, based on electron microscopy analysis (*94*), whereas the forebrain ependymal layer consists almost exclusively of cuboidal ependymal cells (denoted E1 and E2 cells) (*104*). Interestingly, the neural stem cells (type B cells) in the subventricular zone protrude through the layer of cuboidal ependymal cells and contact the ventricle on its apical side with a primary cilium and extend a basal process to a blood vessel, in many ways resembling the morphology of tanycytes (*104*). The ependymal layer in the neurogenic third ventricle has a gradient of cuboidal ependymal in dorsal regions and tanycytes in ventral regions (*98*). The neurogenic potential in the hypothalamic ependymal layer is exclusively found in tanycytes (*39, 40, 42, 43, 98, 183*). Tanycytes in the third ventricle have been studied for decades. Morphological studies have mapped and defined subpopulations of tanycytes according to their position in the ventricle wall and where their basal process projects (*43, 98, 184*). There are at least four different tanycytic subpopulations described in the literature and they are heterogeneous in marker expression (e.g. GFAP, FGF10, FGF18 and Glast) and stem cell potential (*39,*

40, 42, 43, 98, 183). Ependymal cells in the spinal cord also display heterogeneity in expression of markers associated with immature neural cells such as BLBP, RC1, Nestin and CD15 (*94, 95, 102*). Other markers that have a suggested expression in ependymal subpopulations include the astrocyte and neural stem cell marker GFAP, the neural cell adhesion molecule PSA-NCAM and the cannabinoid receptor CB-1 (*92, 95*). The heterogeneity in stem cell/immature marker expression suggests a potential functional heterogeneity within different subpopulations of ependymal cells.

1.5.4 Spinal cord ependymal cells are functionally heterogeneous

Ependymal cells are latent neural stem cells in the adult spinal cord (Paper I) (*94*). The stem cell properties of ependymal cells have been assessed on a population level since the vast majority of ependymal cells are recombined in the FoxJ1-CreER line (Paper I) (*94*). One intriguing question is if all ependymal cells have stem cell properties or if this is contained to a subpopulation of ependymal cells?

To assess the stem cell potential of different ependymal cell subpopulations we used two different CreER mouse lines on Cre-reporter background (R26R-YFP or R26R-tdTomato; Figure 1). The first mouse line expressed CreER under the Glast promoter (Slezak et al., 2007). The Glast-CreER mouse recombines type A pericytes and a small subpopulation of ependymal cells (approximately 10% of all pericytes and 3.5±1.7% of ependymal cells, respectively, Mean±SEM, Paper III) (*109*). Recombined Glast-CreER cells are concentrated in the dorsal region of the ependymal layer, which is also where proliferating ependymal cells are most numerous under physiological conditions (Paper III) (*91, 93*). To investigate if recombined Glast-CreER cells proliferate, mice received BrdU continuously for 3 weeks before analysis. We found that recombined Glast-CreER cells do proliferate, but not more frequently than the average of all ependymal cells (Paper III), demonstrating that the Glast-CreER subpopulation is not the only proliferating subpopulation in the ependymal layer. Proliferation within the ependymal layer is always in close proximity to the surrounding vasculature (Hamilton et al., 2009) and interestingly, the vast majority of Glast-CreER cells extended basal processes that often ended on blood vessels (Paper III). This could suggest an important role for Glast-CreER within the spinal cord stem cell niche, where blood vessels are one important component (*93, 95*). To investigate the *in vitro* neural stem cell potential

of recombined Glast-CreER cells, neurospheres were generated and the recombination assessed over several passages. We found that the recombined Glast-CreER ependymal subpopulation was highly enriched for generating neurospheres. Recombination went up from 1.6±0.2% of ependymal cells in biopsies, to 34.3±3.8% of primary neurospheres in culture (mean±SEM for n=5 animals, Paper III). However, the recombination rate decreased rapidly as the neurospheres were passaged and by the 4th passage almost no neurospheres were recombined (0.6±0.3% recombination at passage 4, mean±SEM, Paper III). This demonstrated that neurospheres derived from Glast-CreER ependymal cells have limited self-renewing capacity and may better be described as progenitor cells. Two previous studies have used the human GFAP promoter to fate map and study one ependymal cell subpopulation. They report similar morphology, marker expression and, most importantly, the same limited *in vitro* self-renewal potential for their ependymal cell subpopulation as for Glast-CreER ependymal cells (Paper III) (*95, 185*). This suggests that cells that are labeled using the human GFAP promoter and recombined Glast-CreER cells might be the same, or overlapping, progenitor populations (Paper III) (*95, 185*).

Spinal cord injury induces a dramatic increase in proliferation throughout the injured segment (*51, 80, 110*). Horky and colleagues investigated the role of dividing versus quiescent parenchymal progenitors acutely after an injury. They wanted to determine if the dividing progenitor pool was responsible for posttraumatic gliogenesis or if quiescent progenitors were recruited. They pre-labeled dividing cells with BrdU and characterized the injury response 24 hours after injury. They found that the vast majority of pre-labeled dividing progenitors died or remained post-mitotic (*80*). We found that ependymal cells act in a similar way when we analyzed animals that had received BrdU for 4 weeks before a dorsal funiculus incision. We could not find a single proliferating ependymal cell pre-labeled with BrdU 4 days after the injury (Paper III). In addition, we found that the location of proliferating ependymal cells within the ependymal layer shifted from the dorsal region during physiological conditions, to the ventral region after injury (Paper III). Together these findings suggest that quiescent cells are recruited to proliferate in the acute phase after spinal cord injury. To assess if Glast-CreER ependymal cells are proliferating after injury, we performed dorsal funiculus incisions and analyzed the animals 7 days later. We were not able to find any proliferating Glast-CreER ependymal cells, nor were they migrating towards the injury site or in any other way reacting to the injury (Paper III).

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In summary, our results suggest that the Glast-CreER ependymal subpopulation represent restricted progenitors, which proliferate under physiological conditions, but which neither display *in vitro* neural stem cell properties nor contribute to scar formation or remyelination in response to injury (Figure 6, Paper III).

The second mouse line used in paper III was the Troy-CreER mouse. Troy is a Tumor necrosis factor receptor family member, which has been implicated in stem cell activity in other organs (*186*). The Troy-CreER mouse recombines a small subpopulation of ependymal cells (approximately 6% of ependymal cells, Paper III) and a subset of pericytes that do not generate progeny or leave the vessel wall after spinal cord injury (data not shown). The vast majority of recombined Troy-CreER ependymal cells have tanycyte-like processes contacting blood vessels, however they are completely quiescent under physiological conditions (Paper III). To assess the *in vitro* neural stem cell potential of recombined Troy-CreER cells, neurospheres were generated and recombination was assessed over several passages. We found similar recombination rates in ependymal cell biopsies as in primary neurosphere cultures (6.7±1.2% and 7.7±1.5% recombination of ependymal cells and neurospheres, respectively, mean±SEM for n=4, Paper III). However, contrary to what was seen for recombined Glast-CreER neurospheres, the recombination rate kept on increasing as the Troy-CreER neurospheres were passaged. By the 4th passage most neurospheres from Troy-CreER mice were recombined (87.8±2.7% recombination at passage 4, mean±SEM for n=4, Paper III). This demonstrated that neurospheres derived from recombined Troy-CreER cells have sustained self-renewal potential over time in culture suggesting that Troy-CreER ependymal cells account for close to all the *in vitro* neural stem cell potential in the ependymal layer and thus the entire adult spinal cord (Figure 6, Paper III).

Next we wanted to investigate if recombined Troy-CreER ependymal cells contribute to scar formation and generation of oligodendrocytes after an injury. We found that quiescent Troy-CreER ependymal cells started to proliferate after injury and gave rise to progeny that migrated towards the lesion site where they differentiated into astrocytes (Figure 6, Paper III). In addition, we found mature recombined oligodendrocytes in Troy-CreER mice 3.5 months after injury, demonstrating that the
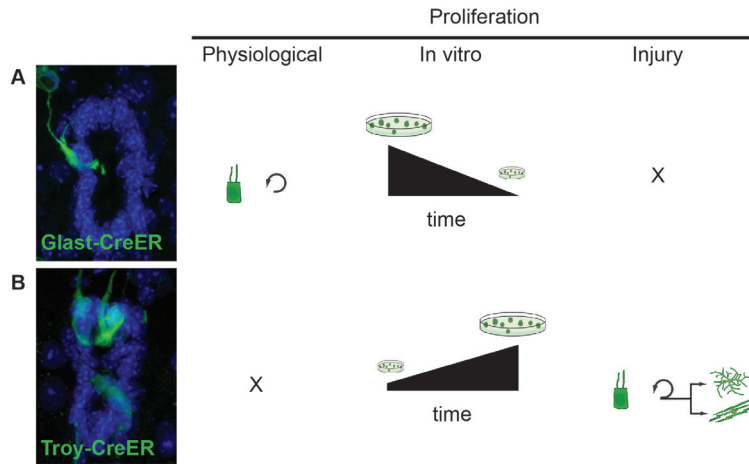
Figure 6. Ependymal cells are functionally heterogeneous.

A. Recombined Glast-CreER ependymal cells (green) proliferate under physiological conditions, have limited self-renewal capacity *in vitro*, but do not give rise to progeny after spinal cord injury.
B. Recombined Troy-CreER ependymal cells (green) are quiescent under physiological conditions, but display stem cell properties with extensive self-renewal *in vitro* and give rise to both scar-forming astrocytes and oligodendrocytes *in vivo* after spinal cord injury. Based on data from Paper III. *Adapted from (108)*

Troy-CreER ependymal subpopulation consists of multipotent cells that can generate both astrocytes and oligodendrocytes.

In paper III we identified functionally distinct subpopulations of ependymal cells using fate mapping in two different mouse lines. Glast-CreER ependymal cells represents a subpopulation of restricted progenitors that proliferates under physiological conditions but have limited self-renewal capacity *in vitro*, and do not give rise to progeny after spinal cord injury. Troy-CreER ependymal cells are latent neural stem cells, which are completely quiescent in the intact spinal cord, but proliferate and give rise to a large number of progeny both *in vitro* and in response to injury. In summary, spinal cord ependymal cells are functionally heterogeneous with proliferating progenitors and quiescent, latent neural stem cells.

2 CONCLUSIONS AND PERSPECTIVES

The main objective of this thesis was to identify spinal cord neural stem cells and investigate their functional role after spinal cord injury.

2.1.1 Conclusions

Specifically we found that:

1. Ependymal cells are latent neural stem cells in the adult spinal cord, which are activated *in vitro* and upon spinal cord injury.

2. Ependymal cells restrict tissue damage and neural loss after spinal cord injury.

3. Spinal cord ependymal cells are functionally heterogeneous, including both restricted progenitor cells and quiescent stem cells.

2.1.2 Ependymal cells are latent neural stem cells in the adult spinal cord

Most studies using cell fate mapping within the injured spinal cord have focused on following the progeny of one specific population of dividing cells. To provide an integrated view of the endogenous cell response, we have followed the three main proliferating neural cell populations in parallel: oligodendrocyte progenitors, resident astrocytes and ependymal cells. Our main findings are that these cells generate progeny that occupy complimentary domains in the injured spinal cord and that resident astrocytes and oligodendrocyte progenitors are lineage restricted progenitors. Moreover, we identify ependymal cells as latent multipotent neural stem cells, which can generate both scar-forming astrocytes and oligodendrocytes after injury. This is similar to what is seen in regenerating species, such as urodele amphibians, where radial glia-like ependymal cells act as neural stem cells (*99*).

Our results are based on incision injuries, specifically in the dorsal funiculus, which is quite mild and unimpairing. This injury model has many advantages when studying endogenous cellular responses like migration and activation since it is a localized injury. However, this is not the best clinically relevant injury model since most humans with spinal cord injury have contusion injuries. Ependymal cells do, indeed, respond to contusion injuries in a similar way as to incision injuries (*187*) (unpublished observations), which validates our model for basic cell fate mapping.

The role of ependymal cells in humans needs to be studied further. Although it is known that adult humans have neurosphere-forming cells in the spinal cord, it is not known if these cells are ependymal cells (*188*). When ependymal cell-progeny or type-A pericyte-progeny is blocked after injury, cyst formation occurs in some mice (Paper II) (*109*). Cyst formation does typically not occur in mice, but is frequent in rats and humans after spinal cord injury (*165*). One intriguing thought is if these cysts could be the consequence of inefficient recruitment of ependymal cells or type-A pericytes to the lesion. If we learn more about how ependymal cells and type-A pericytes interact in scar formation in mice, we might get more insights into how we might be able to prevent cyst formation in rats and humans.

Another intriguing question is to which extent endogenous stem cells are recruited after lesion or disease? In order to answer that question one could look into other types of injuries in the spinal cord such as multiple sclerosis (MS), to define differences and similarities in cellular responses and environmental cues. MS is a demyelinating inflammatory chronic disease that can be studied in EAE mice. There is little evidence to support that ependymal cells are reacting to EAE, but previous studies have only assessed proliferation activity (*187*) or utilized a fate mapping strategy that only includes a small subset of ependymal cells (*189*). It would be interesting to further investigate if ependymal cells are recruited in EAE or any other demyelinating injury or condition.

2.1.3 Ependymal cells restrict tissue damage and neural loss after spinal cord injury.

Glial scarring has traditionally been seen as something bad to overcome after a spinal cord injury, in Paper II we show that scarring by ependymal cells has beneficial effects on recovery. This is in line with some similar studies using GFAP/Vimentin/Nestin promoter elements or knockouts to block or kill reactive astrocytes (*112-115, 159, 190*). However, since these studies target two distinct reactive astrocyte populations at the same time (derived from ependymal cells or resident astrocytes, respectively) it is hard to draw definite conclusions and the results have at times appeared contradicting. In Paper II, we were able to characterize distinct beneficial functions of ependymal-derived astrocytes using the FoxJ1-rasless line. Göritz and colleagues assessed the functional role of type-A pericyte-derived fibrotic cells, using a Glast-rasless line (*109*).

They found that the fibrotic core of the scar is important to seal a lesion (*109*). Before being able modulate the scar in a beneficial way it is crucial to get a more complete view of the role of the distinct domains of the scar (Figure 3). For this purpose it would be valuable to characterize the injury response in an astrocyte-specific rasless mouse where the function of reactive astrocytes can be specifically studied without contamination from ependymal cell progeny. Depending on what these studies show, it might be desirable to modulate the stromal core together with non-ependymal-derived reactive astrocytes as a future therapeutic approach. Stromal cells derived from type-A pericytes are required to seal the lesion, but it has been suggested that partial inhibition of these cells might have beneficial effects after injury (*109, 191*). Therefore, a combinatorial treatment strategy with partial inhibition of both these cell populations might be beneficial for recovery.

The beneficial effects of ependymal cells after an injury are most likely not only due to their cell replacement properties by generating scar tissue and remyelinating oligodendrocytes. In paper III, we show that ependymal derived cells are a major source of neurotrophic support, which makes it likely that some beneficial effect is from modulating the injury environment to make is less toxic and this could explain why we see an increased neuronal loss without ependymal cell progeny. However, the mechanism behind the neurotrophic effect on neurons is not clear. One way to identify the neurotrophic factor(s) involved in this process is to utilize FoxJ1-rasless mice and then treat them with different factors in order to rescue the deleterious phenotype.

We have several candidate neurotrophic factors, such as IGF-1, HGF and CNTF which are highly expressed in ependymal cells and ependymal–derived cells 7 days after injury (unpublished observations). Ependymal cells are still expressing these factors 14 days after injury, but then more distributed within the parenchyma (Paper II). It is possible that ependymal cells distribute these factors to the surrounding tissue.

It is tempting to speculate that the close interplay between ependymal cells and CSF plays a role in regulating their proliferative v/s quiescent state. We see a significant increase in proliferation of ependymal cells in segments distant from a spinal cord injury (Paper III), which possibly could be explained by increasing levels of growth factors in the CSF. Indeed, infusion of growth factors (bFGF and EGF) into the 4th

ventricle induces proliferation of spinal cord ependymal cells (*106*). It would be very informative to analyze the levels of growth factors in CSF after an injury to get insights into how endogenous at all levels of the central nervous system cells are being affected by an injury. Moreover, many transplantation studies have been shown to lead to trophic support by expression of various growth factors. However, the effect on proliferation and differentiation of resident stem cells (ependymal cells) has often been overlooked or not studied at all.

2.1.4 Spinal cord ependymal cells are functionally heterogeneous

We have characterized ependymal cell injury response and the functional implication of ependymal cell activation and scarring (Paper I and Paper II). However, in order to be able to modulate the injury response to promote recovery one needs to characterize ependymal cells further. Troy-CreER cells represent a small subpopulation of quiescent ependymal cells that can generate both astrocytes and oligodendrocytes after injury. This raises the question if individual ependymal cells are multipotent *in vivo* and if the same ependymal cells generate scar-forming astrocytes and oligodendrocytes? To address these questions one could do *in vivo* clonal cell analysis of individual ependymal cells.

Decarolis and colleagues recently reported functional heterogeneity of radial glial-like neural stem cells in the hippocampus using the Nestin-CreER and Glast-CreER lines. They show that both cell populations represent neurogenic stem cells and propose that Glast-CreER cells are "dormant" under physiological conditions but that they are the cells that respond to injury (*179*). This is similar, although molecularly different, to what we find in Paper III; quiescent Troy-CreER cells respond to spinal cord injury, whereas mitotic Glast-CreER cells do not. These two niches should be further evaluated in the sense of harboring two types of progenitor/stem cells that are functionally heterogeneous. In addition, investigating differences and similarities between physiological and injury-induced proliferation in these regions could provide valuable insights to stem cell maintenance and activation.

Several pieces of evidence suggest that tanycytes are the ependymal cell type with stem/progenitor cell properties, both in the hypothalamus and spinal cord, and that cuboidal ependymal cells might have other important functions, such as propulsion of

CSF flow (*90*). Cuboidal ependymal cells might constitute post-mitotic niche cells (as they do in the lateral ventricle wall) whereas tanycytes are cells that signals between the CSF and parenchyma and a sub population of tanycytes could, in addition, be proliferating or quiescent neural stem cells. In the future, it would be interesting to compare the transcriptional profile of different subtypes of tanycytes and cuboidal ependymal cells in the spinal cord to get more insights about their functional differences and roles. This would provide a better understanding of the need and utility of having both limited progenitor cells that maintain homeostasis versus quiescent stem cells that are activated only upon injury. It ultimately comes down to the intriguing questions of how we should define stem cells when stemness is transient and dependent on context. One cell can act as a stem cell in one setting but not at all in another, whereas cues that do not affect the already active stem cell pool can activate a dormant cell. Once we understand these concepts better we might be able to induce and modulate cellular responses in a beneficial way to promote recovery after spinal cord injury.

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