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NEURAL STEM CELLS IN THE SPINAL CORD - IDENTITY, FUNCTION AND POTENTIAL

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**Karolinska
Institutet**

Stockholm 2020

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Published by Karolinska Institutet.

Printed by Universitetservice US AB

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ISBN 978-91-7831-948-0

Neural stem cells in the spinal cord - Identity, function and potential

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

'I recall seeing a package to make quotes'

– Snowball, StackExchange

ABSTRACT

A large body of work has been dedicated to understanding and remedying the pathological processes that take place after spinal cord injury. Despite these efforts, treatment options are limited and patients with spinal cord injury are left with life-long disability.

The work described in this thesis is part of an ongoing effort to learn about endogenous neural stem cells as potential targets for therapies aiming to repair the injured spinal cord.

In **paper I**, we studied the effects of ablating the ependymal neural stem cell response to spinal cord injury, in order to understand their functional role in the injury response. We found that the ependymal cell contribution to the injury response keeps the injury from growing deeper and severing additional axonal tracts, as well as limits neuronal loss. These findings identify ependymal cells as an interesting target for intervention.

In **paper II**, we discovered a latently accessible program for oligodendrogenesis in ependymal cells by analyzing their gene expression and chromatin accessibility patterns. We activated the oligodendrogenesis program using a combination of forced Olig2 expression and spinal cord injury. The activation of the oligodendrogenesis program leads to an increased production of ependymal-derived oligodendrocytes, enhances remyelination and supports conduction of nerve impulses in the corticospinal tract after spinal cord injury.

In **paper III**, we identified a subset of ependymal cells – EpA cells – that harbors the *in vitro* stem cell capacity of the spinal cord and responds to spinal cord injury by both self-duplicating and generating migrating progeny that differentiates into scar-forming astrocytes and oligodendrocytes. By performing single cell RNA sequencing we found that shortly after injury, the highly differentiated EpA cells dedifferentiate to acquire a stem cell-like state.

The work presented in this thesis supports an important role of ependymal cells in the response to spinal cord injury, and explores their plasticity as well as the manipulation of their lineage fate choices. Lastly, it describes the activation of a subgroup of ependymal neural stem cells with the ability to respond to spinal cord injury. Through these studies, we have identified and characterized a population of resident neural stem cells that represents a promising therapeutic target to treat spinal cord injury.

LIST OF SCIENTIFIC PAPERS

- I. Hanna Sabelström, **Moa Stenudd**, Pedro Réu, David O. Dias, Marta Elfineh, Sofia Zdunek, Peter Damberg, Christian Göritz and Jonas Frisé. (2013). Resident Neural Stem Cells Restrict Tissue Damage and Neuronal Loss After Spinal Cord Injury in Mice. *Science*. 342:637-640.
- II. Enric Llorens-Bobadilla, James Chell, Pierre La Merre, Margherita Zamboni, Joseph Bergensträhle, **Moa Stenudd**, Joakim Lundeberg, Marie Carlén and Jonas Frisé. (2020). A latent lineage potential in resident neural stem cells enables spinal cord repair. *Science*. 370, eabb8795.
- III. **Moa Stenudd**, Hanna Sabelström, Enric Llorens-Bobadilla, Margherita Zamboni, Hans Blom, Hjalmar Brismar, Shupe Zhang, Onur Basak, Hans Clevers, Christian Göritz, Fanie Barnabé-Heider and Jonas Frisé. Identification of a discrete subpopulation of spinal cord ependymal cells with neural stem cell properties. *Manuscript*.

Publications not included in this thesis

Moa Stenudd, Hanna Sabelström, and Jonas Frisé. Role of endogenous neural stem cells in spinal cord injury and repair. *JAMA Neurology*, 2015.

Hanna Sabelström, **Moa Stenudd**, and Jonas Frisé. Neural stem cells in the adult spinal cord. *Experimental Neurology*, 2014.

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LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
AIS	ASIA Impairment Scale
ASIA	American Spinal Cord Injury Association
BMS	Basso Mouse Scale
CFP	Cyan Fluorescent Protein
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CSPG	Chondroitin Sulfate Proteoglycan
CX30	Connexin 30
ECM	Extracellular Matrix
FACS	Fluorescence Activated Cell Sorting
FOXA2	Forkhead Box Protein A2
FOXJ1	Forkhead Box Protein J1
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
HGF	Hepatocyte Growth Factor
IGF1	Insulin-like Growth Factor 1
JAK	Janus Kinase
MBP	Myelin Basic Protein
MSC	Mesenchymal Stem Cell
MYRF	Myelin Regulatory Factor
NSC	Neural Stem Cell
NG2	Neural/Glial Antigen 2
OEC	Olfactory Ensheathing Cell
OLIG2	Oligodendrocyte Transcription Factor 2
OPC	Oligodendrocyte Progenitor Cell
PDGFR α	Platelet-Derived Growth Factor Receptor alpha
PNS	Peripheral Nervous System
RFP	Red Fluorescent Protein
SCI	Spinal Cord Injury

SGZ	Subgranular Zone
SOCS3	Suppressor of Cytokine Signaling 3
SOX10	SRY-Box Transcription Factor 10
STAT	Signal Transducer and Activator of Transcription
SVZ	Subventricular Zone
TNFRSF19	Tumor Necrosis Factor Receptor Superfamily Member 19
TGF β	Transforming Growth Factor beta
YFP	Yellow Fluorescent Protein

1 INTRODUCTION

Traumatic spinal cord injuries are life-changing and biologically complex. Millions of people worldwide live with long-term disabilities such as motor deficits, loss of bladder control and neuropathic pain after spinal cord injury (SCI) (Ahuja et al., 2017; James et al., 2019). Because of the significant suffering and care need of SCI patients, considerable research efforts have focused on developing treatments promoting spinal cord repair. Despite these efforts, there is still no cure, and treatment options are largely limited to managing symptoms, such as pain (Widerström-Noga, 2017).

The spinal cord is organized into grey matter containing nerve cell bodies, and white matter containing myelinated nerve fibers, axons. The axons connect the brain with the body and relay sensory and motor signals (Ahuja et al., 2017). Traumatic SCI typically results in cut off nerve fibers accompanied by massive cell death. The injury is followed by a considerable immune reaction, and an invasion and generation of new cells at the injury site. Understanding the role of the arriving and newly formed cells in the lesion is of utmost importance to understand how to treat SCI.

Following the initial injury reaction, a scar is formed at the lesion site. The scar consists of a fibrotic core infiltrated with inflammatory immune cells, that is surrounded by layers of newly formed astrocytes derived predominantly from neural stem cells (NSCs), as well as reactive resident astrocytes, and some neural/glial antigen 2 (NG2)-expressing oligodendrocyte progenitor cells (OPCs) (Bradbury & Burnside, 2019; Sabelström, Stenudd, & Frisé, 2013). The scar is thought to have both a positive effect on the outcome of the injury by stabilizing the tissue and controlling the spread of the inflammatory immune reaction, and a negative effect on the outcome by blocking regrowth of nerve fibers through the injury site (Bradbury & Burnside, 2019). One goal of the work presented in this thesis is to understand the role of the NSC-derived cells that populate the scar.

A multitude of studies have focused on achieving regeneration of lost or damaged spinal cord tissue (Assinck, Duncan, Hilton, et al., 2017; Barnabé-Heider & Frisé, 2008; Bradbury & Burnside, 2019). Many attempts have involved transplanting stem/progenitor cells into the spinal cord lesion site. However, transplantation is typically an invasive process with serious disadvantages connected to both practical aspects such as the availability of cells to transplant, and to our immune system's reaction to cells from exogenous sources (discussed further in section 4).

The manipulation of endogenous cells may provide an elegant solution to problems associated with cell transplantation. Utilizing cells that are already present in an injured tissue to repair it does not require invasive procedures or introduce exogenic material to the injured tissue.

In order to manipulate endogenous stem cells to, for instance, expand their population size or alter their fate choices, it is important to identify candidate neural stem cell populations that can

be targeted for intervention and to understand their current functions and the mechanisms that control them.

By focusing on these three areas; the function (Paper I), the plasticity (Paper II), and the identity (Paper III) of stem cells in the spinal cord, I hope that the work in this thesis can bring us one step closer to help the spinal cord repair itself with resident cells.

2 REACTION AND REPAIR FOLLOWING SPINAL CORD INJURY

A traumatic insult to the central nervous system is initially mechanical. It causes structural damage to neurons and their axons, and the death of neurons and glial cells. The primary insult is followed by a cascade of events that leads to both secondary damage and to the sequestration of the injured area from surrounding tissue through the formation of a scar (Ahuja et al., 2017; Burda et al., 2016).

This chapter of the thesis will focus on the reaction to traumatic injury and subsequent scarring in the adult mammalian spinal cord. The process is similar to the one following traumatic injury in the mammalian brain, but there are some differences. For instance, the inflammatory response and astrocyte reactivity following spinal cord injury is more widespread compared to after traumatic brain injury, and different cell types harbor the stem cell capacity of the spinal cord and the brain (Bradbury & Burnside, 2019; Kriegstein & Alvarez-Buylla, 2009; Sabelström, Stenudd, & Frisén, 2013; Schnell, 1999) [stem cell identities are covered in chapter 3].

2.1 ACUTE AND SUBACUTE REACTION TO SPINAL CORD INJURY

A cascade of events that precede scar formation start shortly after a traumatic SCI. The acute period after the injury is characterized by bleeding and local edema (Bradbury & Burnside, 2019). Already at this early time point, many cells die as a consequence of secondary changes directly following the injury, such as cell permeabilization, presence of pro-apoptotic signals and ischemia resulting from a disrupted microvascular blood supply to the lesion area. Severe swelling, which can result from strong inflammation or disruption of the blood-spinal cord barrier, can lead to additional compression damage to the spinal cord (Ahuja et al., 2017).

Cell damage, cell death and tissue necrosis lead to the activation of microglia in the lesion site, starting within minutes after SCI (Jin & Yamashita, 2016; Kroner & Rosas Almanza, 2019). The lesion site is subsequently infiltrated by other immune cells such as activated macrophages, lymphocytes and neutrophils. Immune cells clear up myelin debris and cell residues from the lesion core, but they also release cytotoxins that can generate secondary damage (Kigerl et al., 2009; X. Liu et al., 2018; Tran et al., 2018).

Glial cells like astrocytes (resident and ependymal cell-derived) and oligodendrocyte progenitor cells (OPCs), as well as pericyte-derived fibrotic cells are recruited to the lesion area within days after SCI. These cells play important roles in modulating the environment after SCI, and they form the glial scar that is described in the next section (Barnabé-Heider et al., 2010; Göritz et al., 2011; Shea et al., 2017).

After an axon is severed, axonal dieback of the proximal end of the cut will start within half an hour, while the distal end will undergo a similar process, called Wallerian degeneration, at a later time point (Kerschensteiner et al., 2005). Axons attempting to regrow are found less than a day after injury, but they fail to grow in an organized manner and do not pass the glial scar (Kerschensteiner et al., 2005). The failure to regrow is likely a result of a combination of

environmental factors and the neurons intrinsic capacity to regrow its axon (Bradbury & Burnside, 2019; Kerschensteiner et al., 2005).

The neurons' intrinsic ability to regenerate their axons is far greater in the peripheral nervous system (PNS) compared to in the CNS (Hilton & Bradke, 2017). A regenerating axon in the PNS will form a growth cone, which is a structure that contains microtubules organized in the directionality of the cut axon (Ertürk et al., 2007; Hilton & Bradke, 2017). The growth cone is instrumental for axonal regeneration, and axons cut in the CNS fail to form one (Bradke et al., 2012). Instead, the tip of a cut axon in the CNS forms a retraction bulb, in which microtubules are disorganized (Ertürk et al., 2007).

Factors in the local environment that limit regrowth of axons include changes in tissue architecture following injury and scarring, as well as the local microenvironment in the lesion area [discussed in section 2.3].

2.2 SCARRING AFTER SPINAL CORD INJURY

A scar is formed at the site of injury. While it is traditionally called the glial scar, it inhabits both glial and non-glial cell types. The scar consists of a core with fibroblast-like cells and inflammatory immune cells. The scar core is immediately surrounded by a layer of astrocytes, in part generated from ependymal cells, which in turn is surrounded by hypertrophic resident astrocytes and some NG2-expressing OPCs in the perimeter of the scar (Barnabé-Heider et al., 2010; Bradbury & Burnside, 2019; Cregg et al., 2014; Göritz et al., 2011; Li et al., 2018; Meletis et al., 2008; Soderblom et al., 2013). Scar formation is a dynamic process. The glial scar matures with time, and many of the components that start arriving early after injury reach maximal densities weeks later. In most mammalian species, a cyst is formed in the center of the scar (Bradbury & Burnside, 2019).



Figure 1 | Glial & fibrotic compartments of the glial scar. Illustration of the distribution of glial scar components from different sources. In yellow, type A pericyte-derived fibroblast-like cells in the scar core. In green, ependymal-derived astrocytes. In red, astrocyte scar component derived from resident astrocytes.

Illustration from Stenudd et al., 2015. Used with permission from the American Medical Association.

The fibroblast-like cells that populate the scar core are mainly generated from type A pericytes. Type A pericyte-derived cells peak at around two weeks after injury (Göritz et al., 2011). The fibrotic scar is important for the maintenance of tissue integrity after SCI, but also plays a role in blocking axonal regrowth (Dias et al., 2018; Göritz et al., 2011).

Local and infiltrating immune cells inhabit the scar, and the infiltration of immune cells peaks at four weeks after injury (Bellver-Landete et al., 2019; Kigerl et al., 2009). In addition to fibroblast-like cells and inflammatory immune cells, the scar core contains extracellular matrix (ECM) deposits, as well as glycoproteins such as chondroitin sulfate proteoglycans (CSPGs) (Shea et al., 2017).

The astrocytic component of the glial scar is made up of newly formed astrocytes surrounding the lesion core, and reactive astrocytes in the perimeter of the scar. Astrocytes close to the lesion core have elongated morphologies, and they come from recently divided cells. Further away from the lesion core, the astrocytic scar component consists of hypertrophied, stellate resident astrocytes (Barnabé-Heider et al., 2010; Wanner et al., 2013). Newly formed astrocytes are more frequently generated from ependymal cells compared to resident astrocytes (Barnabé-Heider et al., 2010). The density of glial fibrillary acidic protein (GFAP)-expressing astrocytes only increases around 2- to 4-fold after injury compared to uninjured tissue (Ertürk et al., 2012). However, this may not give the full picture of the astrocyte population growth, since astrocytes derived from ependymal cells do not uniformly express GFAP (Meletis et al., 2008).

2.3 DUAL ROLES OF ASTROCYTES AND IMMUNE CELLS IN THE GLIAL SCAR

Immune cells and astrocytes in the scar appear to have both beneficial and detrimental effects after SCI.

The injury reaction by microglia and macrophages plays an important role in the recovery after SCI by cleaning up residues from dead cells and myelin debris from the lesion core, but they can also contribute to increased inflammation and neuronal death (Kigerl et al., 2009; X. Liu et al., 2018; Tran et al., 2018). These opposing effects seem to be carried out by different subgroups of microglia and macrophages.

Microglia and macrophages can be subdivided into M1 and M2 subtypes depending on the roles they play in the injury response (Kigerl et al., 2009). M1 macrophages are pro-inflammatory and neurotoxic, while M2 macrophages act anti-inflammatory and more effectively clear necrotic debris. Unfortunately, the M2 response is smaller and lasts for a shorter time compared to the M1 macrophage response (Kigerl et al., 2009). Similar to M1 macrophages, M1 microglia are neurotoxic and release pro-inflammatory cytokines, while M2 microglia act anti-inflammatory (X. Liu et al., 2018).

The elongated astrocytes surrounding the lesion core contract the scar and function as a border, hindering the spread of inflammatory cells from the lesioned tissue to surrounding healthy spinal cord tissue and thereby reducing secondary injury (Cregg et al., 2014; Faulkner et al., 2004; Herrmann et al., 2008; Okada et al., 2006; Sabelström, Stenudd, Réu, et al., 2013; Wanner et al., 2013).

At the same time, a role of glial scar astrocytes in forming a barrier to axonal regrowth has been extensively studied in both brain and spinal cord lesions.

Molecules in the ECM of the scar, including proteoglycans, ephrins, semaphorins and myelin-associated proteins (such as Nogo-A), play a role in blocking axonal regrowth (Griffin & Bradke, 2020; Schwab & Strittmatter, 2014). Chondroitin sulfate proteoglycans, or CSPGs, are expressed by reactive astrocytes as well as other cell types in the scar, such as fibrotic cells in the core of the scar. CSPGs can bind to cell surface receptors on neurons and block neurite outgrowth (Davies et al., 1999; Fisher et al., 2011; Friedlander et al., 1994; Göritz et al., 2011; McKeon et al., 1999; Shen et al., 2009).

The view of the glial component of the scar as an obstacle to regeneration may be reductionist. A study by Anderson et al. (2016) suggested that astrocytes support axonal regrowth by creating a permissive environment/bridge, for instance by producing growth-promoting CSPG5. Tran et al. (2018), perhaps in an attempt to consolidate the opposing views on the effects of the astrocytic scar on axonal regrowth, argue that the role of astrocytes as either a barrier or a bridge for axonal regeneration may be dependent on local environmental cues, such as the degree of inflammation in the lesion area.

The, still ongoing, discussion with evidence for both positive and negative effects of the astrocytic scar component on recovery from SCI led us to examine the role of the ependymal cell generated glial scar component in **Paper I**.

2.4 DE- AND REMYELINATION AFTER SPINAL CORD INJURY

Oligodendrocytes myelinate axons, which is important for neuronal survival and to increase the conduction speed of nerve impulses. Oligodendrocytes are sensitive to the microenvironment after SCI, and as a result there is a massive loss of oligodendrocytes for days after injury (Giacci et al., 2018; X. Z. Liu et al., 1997; F. Sun et al., 2010). The loss of myelinating oligodendrocytes is accompanied by demyelination of axons (Pukos et al., 2019; Rabchevsky et al., 2007).

OPCs are identified by their expression of NG2 and platelet-derived growth factor alpha (PDGFR α), and constitute an important source of new myelinating oligodendrocytes after spinal cord injury (Assinck, Duncan, Plemel, et al., 2017; Hall et al., 1996; Pringle et al., 1992). OPCs proliferate to self-renew and generate oligodendrocytes both in the intact spinal cord and after injury (Assinck, Duncan, Plemel, et al., 2017; Barnabé-Heider et al., 2010; Duncan et al., 2020; Horner et al., 2000; Kang et al., 2010; F. Sun et al., 2010; Zhu et al., 2011). After injury, they give rise to myelinating oligodendrocytes and some Schwann cells (Assinck, Duncan, Plemel, et al., 2017; Barnabé-Heider et al., 2010; Kang et al., 2010). The production of new oligodendrocytes from OPCs continues for months after injury (Hesp et al., 2015). A fate mapping study by Assinck et al. (2017) reported that approximately 30% of new myelin sheets found in a lesion three months after SCI were generated by oligodendrocytes produced by OPCs after the injury. A small portion of remyelinating oligodendrocytes are also produced from ependymal cells (Barnabé-Heider et al., 2010; Meletis et al., 2008).

Remyelination is an important goal for therapies aiming to improve functional outcome after spinal cord injury. As a result, improving the availability of remyelinating cells is a goal of many

therapeutic efforts. In **paper II**, we examine the feasibility and effects of increasing the oligodendrocyte generation by ependymal cells.

3 NEURAL STEM CELLS IN THE ADULT CENTRAL NERVOUS SYSTEM

When discussing the identities of stem cells, it seems appropriate to start by considering what a stem cell is. Traditional definitions of stem cells typically focus on two areas: multipotency, which is a cell's ability to generate cells of different types, and self-renewal, which indicates a cell's ability to self-duplicate to generate new multipotent stem cells (Gage, 2000).

For years, the prevailing view was that no new nerve cells are born in the adult central nervous system. However, evidence of adult neurogenesis has slowly accumulated since the 60's, when Joseph Altman and Gopal Das found new-born neurons in the olfactory bulb and dentate gyrus in the hippocampus of adult rats (Altman, 1962; Altman & Das, 1965). Initially, the work by Altman and Das did not attract large interest and the research efforts in this field were limited. Almost two decades later, new-born neurons that integrated into functional circuits were found in adult canary birds (S. A. Goldman & Nottebohm, 1983).

The interest in the regenerative abilities of the mammalian CNS grew in the early 90's, when a few research groups showed that it's possible to isolate NSCs from the brain of adult rodents and expand them *in vitro* (Reynolds & Weiss, 1992; Richards et al., 1992). In the late 90's, elegant work by Eriksson et al. (1998) showed that new nerve cells are born during adulthood in the human hippocampus. This seminal work has been followed by the characterization of adult neurogenesis in the human brain, where new neurons are born throughout our lives (Ernst et al., 2014; Spalding et al., 2013). The situation is different in the spinal cord, and there is to date no compelling evidence supporting neurogenesis in the adult spinal cord.

NSCs are present in both the brain and the spinal cord, and the potential of using their capacity to repair injuries in the central nervous system have fascinated researchers during the last decades (Doetsch et al., 1999; Johansson et al., 1999; Meletis et al., 2008; Suh et al., 2007). This chapter will cover the identity and functions of NSCs in the CNS.

3.1 THE BRAIN

The subventricular zone (SVZ) of the lateral ventricle walls, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus are the two main NSC niches in the adult mammalian brain (Bonaguidi et al., 2011; Doetsch et al., 1999; C. Lois & Alvarez-Buylla, 1993; Suh et al., 2007). The NSCs of the adult brain are astrocyte-like cells (Doetsch et al., 1999; Kempermann et al., 2015; Seri et al., 2001).

3.1.1 The subventricular zone

NSCs, sometimes referred to as type B cells, reside in the SVZ of the mammalian brain. Type B cells can generate neurons, astrocytes and oligodendrocytes both *in vitro* and *in vivo* (Calzolari et al., 2015; C. Lois & Alvarez-Buylla, 1993; Menn et al., 2006; Sohn et al., 2015).

The most extensively studied process starting from type B cells is the birth of new neurons. Type B cells cycle between a quiescent and an active (proliferating) state (Basak et al., 2018). Active type B cells divide to self-renew and/or produce rapidly dividing transit-amplifying cells that, in turn, give rise to neuroblasts that migrate to form neurons in the olfactory bulb in mice (Calzolari et al., 2015; Carlos Lois et al., 1996).

Stem cells in the SVZ are functionally heterogeneous. Distinct pools of SVZ stem cells can generate either neurons and astrocytes or oligodendrocytes, while the same cell does not typically generate all three (Ortega et al., 2013). However, lineage plasticity can be unlocked by demyelinating lesions, leading to the generation of oligodendrocytes from NSCs that typically give rise to neurons (Jablonska et al., 2010). Different olfactory bulb interneuron types are generated by different committed NSCs that populate distinct regions of the SVZ (Merkle et al., 2007; Young et al., 2007).

In humans, no new neurons are formed in the olfactory bulb (Bergmann et al., 2012). Instead, neural stem cells in the subventricular zone are a plausible source of adult-born neurons in the striatum (Ernst et al., 2014).

In response to ischemic injury in mice, quiescent type B cells are activated. They transition from quiescence to an active state via a primed quiescent state (Llorens-Bobadilla et al., 2015). During the transition, protein synthesis and cell-cycle mRNA expression increase (Llorens-Bobadilla et al., 2015). After cortical injury, type B cells give rise to astroglial cells in the glial scar (Benner et al., 2013). NSCs in the SVZ generate new neurons in the striatum after ischemic injury (Arvidsson et al., 2002; Yamashita et al., 2006). Although, this response is limited and the number of striatal neurons that are born corresponds to around 1 in 500 lost neurons (Arvidsson et al., 2002). In other words, despite the neurogenic capacities of the region, the injury response in the SVZ leads to a refocus of NSCs towards gliogenesis.

3.1.2 The subgranular zone of the hippocampus

In the SGZ of the dentate gyrus in the hippocampus, radial glia-like cells are the source of adult neurogenesis as well as astrogenesis. Radial glia-like cells generate intermediate progenitor cells that, in turn, give rise to either astrocytes or migratory neuroblasts that mature into hippocampal granule cells (Bonaguidi et al., 2011; Kempermann et al., 2015). Radial glia-like cells in the SGZ do not generate oligodendrocyte lineage cells under physiological conditions, however they can be activated to produce oligodendrocytes by the inactivation of the neurofibromin 1 gene (G. J. Sun et al., 2015). In the human hippocampus, new neurons are born throughout our lives (Spalding et al., 2013).

The process of ongoing neurogenesis in the adult brain has generated substantial research interest, in particular in the hippocampus where new neurons appear to play a role in our ability to learn (Schmidt-Hieber et al., 2004). The radial glia-like cells' reaction to injury is not as extensively studied. However, in response to neural hyperexcitation, which is a hallmark of epilepsy, radial glia-like stem cells in the SGZ are activated and start dividing. Depending on the degree of hyperexcitation, they divide asymmetrically to produce one

intermediary progenitor cell and one astrocyte, or symmetrically to produce two reactive astrocytes. Both of these injury reactions deplete the SGZ radial glia-like population and exhaust adult hippocampal neurogenesis (Sierra et al., 2015).

3.2 THE SPINAL CORD

While functional roles of neural stem- and progenitor cells can be studied in the brain under physiological conditions, it is an uneventful endeavor in the spinal cord where we do not observe any NSC activity under physiological conditions (Barnabé-Heider et al., 2010; Sohn et al., 2015). Instead, functional studies on NSCs in the spinal cord typically focus on *in vitro* stem cell activity, or on the context of injury or disease (Cusimano et al., 2018; Grégoire et al., 2015; Johansson et al., 1999; Lacroix et al., 2014; Li et al., 2016; Meletis et al., 2008; North et al., 2015; Sabelström, Stenudd, Réu, et al., 2013).

Different glial cell types, including astrocytes, NG2-expressing oligodendrocyte progenitor cells (OPCs) and ependymal cells, have been suggested to harbor the NSC potential of the adult spinal cord (Horner et al., 2000; Johansson et al., 1999; Ohori et al., 2006). Fate mapping studies have failed to show differentiation to multiple glial lineages from astrocytes (Sabelström, Stenudd, & Frisé, 2013). Most studies in both young and adult mice support that NG2-expressing OPCs only generate myelinating cells and new OPCs after injury (Assinck, Duncan, Plemel, et al., 2017; Barnabé-Heider et al., 2010; Kang et al., 2010; Zhu et al., 2011). Nonetheless, there is some evidence suggesting that NG2-expressing OPCs may be capable of astrocyte differentiation after SCI (Hackett et al., 2016; Huang et al., 2018). In contrast, there is a multitude of studies showing self-renewal and multilineage differentiation from ependymal cells in the adult spinal cord.

3.2.1 Spinal cord ependymal cells act as neural stem cells after spinal cord injury

Under physiological conditions, ependymal cells do not show stem cell activity. Instead, they remain around the central canal and proliferate slowly to produce new ependymal cells (Barnabé-Heider et al., 2010; Horner et al., 2000; Meletis et al., 2008; Sohn et al., 2015). However, they start proliferating vigorously within 24 hours after SCI (Barnabé-Heider et al., 2010; Beattie et al., 1997; Johansson et al., 1999; Lacroix et al., 2014; Meletis et al., 2008). Around three days after injury, ependymal cell progeny starts leaving the central canal. Migrated ependymal progeny differentiate into both scar-forming astrocytes and oligodendrocyte lineage cells, including myelinating oligodendrocytes. While generating migrating progeny, the ependymal cells are able to self-renew sufficiently to maintain their own population (Barnabé-Heider et al., 2010; Johansson et al., 1999; Li et al., 2018; Meletis et al., 2008; A. Mothe & Tator, 2005; North et al., 2015).

Neurosphere culture experiments point in the same direction. Unlike astrocytes or OPCs, cells from the ependymal cell population divide to efficiently produce neurospheres *in vitro*. Ependymal cell-derived neurosphere cultures generate increasing numbers of neurospheres over multiple passages (Barnabé-Heider et al., 2010; Meletis et al., 2008; Pfenninger et al.,

2011). Neurospheres generated from ependymal cells are multipotent, and can be differentiated into all three neural lineages (astrocytes, oligodendrocytes and neurons) *in vitro* (Barnabé-Heider et al., 2010; Johansson et al., 1999; Meletis et al., 2008). Cells from the ependyma of the injured spinal cord expand faster *in vitro*. They generate more neurospheres, which grow larger, compared to cells from the uninjured spinal cord (Barnabé-Heider et al., 2010; Li et al., 2018; Moreno-Manzano et al., 2009).

In conclusion, multiple lines of evidence support that cells within the ependymal cell population are activated by injury or *in vitro* to act as NSCs in the spinal cord (Barnabé-Heider et al., 2010; Johansson et al., 1999; Li et al., 2018; Meletis et al., 2008; A. Mothe & Tator, 2005).

This means that different cell types carry the NSC capacity of the brain and spinal cord, since ependymal cells do not display stem cell qualities in the brain. Ependymal cells around the lateral ventricles are activated and generate migrating progeny in response to stroke in the adult brain, but they fail to self-renew sufficiently and are depleted in the process (Carlén et al., 2009). Instead, the stem cells of the SVZ in the brain are astrocyte-like type B cells.

Most of the studies cited above contain data on neural stem cells in mice and rats. Less is known about the stem cell potential of ependymal cells in humans. Of note, no cell division has been detected in the human ependyma using antibody staining against the Ki67 antigen, which is expressed by proliferating cells. This implies that ependymal cells in the spinal cord of adult humans either divide very rarely, or not at all (Becker et al., 2018). Meanwhile, efficiently self-renewing and multipotent cells from the human spinal cord can be grown and passaged in neurosphere assays (A. J. Mothe et al., 2011).

While stem cells in the spinal cord respond to SCI and can differentiate into neurons *in vitro*, they are not known to give rise to neurons *in vivo* (Barnabé-Heider et al., 2010). Intriguingly, a study by Shihabuddin et al. (2000) revealed that spinal cord-derived NSCs that have been expanded *in vitro* can generate neurons *in vivo* in neurogenic regions, even if they do not give rise to new neurons in the spinal cord. Upon transplantation of NSCs from the rat spinal cord into the hippocampus, some NSCs derived from the spinal cord gave rise to cells closely resembling hippocampal granular neurons. This demonstrates an inherent plasticity and a sensitivity to the microenvironment surrounding NSCs that may be interesting to explore further in the context of manipulating the ependymal cells' response to SCI.

3.2.2 Characteristics of and heterogeneity within the ependymal stem cell niche in the adult spinal cord

Spinal cord ependymal cells line the central canal. Ependymal cells have motile cilia propelling cerebrospinal fluid, and they form a barrier between the cerebrospinal fluid and the spinal cord parenchyma (Bruni & Reddy, 1987; Cathcart & Worthington, 1964; Del Bigio, 1995).

Ependymal cells in the adult spinal cord are morphologically heterogeneous, with three distinct morphologies that have been identified using electron microscopy. The most common morphology is a multi-ciliated cuboidal cell with no dorsal process. Another common morphology is tanycytic, which describes an ependymal cell with a single cilium, and a single dorsal process. The least common morphological subtype is the radial ependymal cell, which is typically located in the dorsal or ventral pole of the central canal and have a long dorsal process (Bruni & Reddy, 1987; Meletis et al., 2008; Seitz et al., 1981).

Ependymal cells uniformly and, in the spinal cord, exclusively express motile cilia related molecular markers such as forkhead box protein J1 (FoxJ1) (Ghazale et al., 2019; Zeisel et al., 2018). Other molecular markers, such as the immature neural cell-associated proteins brain lipid-binding protein (BLBP) and Nestin, as well as the cannabinoid receptor 1 protein (CB1), are unevenly distributed within the ependymal layer (Garcia-Ovejero et al., 2013; Hamilton et al., 2009; Sabourin et al., 2009).

While most characterization of ependymal cells and their stem cell capacities has been done in rodents, a recent study compared the ependymal layer of mouse and human (Ghazale et al., 2019). A set of 120 transcription factors expressed in ependymal cells in both mouse and human were picked out. Some of these transcription factors are expressed only in a small part of the ependymal layer, such as the ventrally located aristaless related homeobox (ARX) and forkhead box A2 (FOXA2), and the dorsally located msh homeobox 1 (MSX1). This dorsal-ventral patterning was conserved between the species, as well as the expression of some frequently used molecular markers for ependymal cells and their progeny (i.e. Foxj1, Pax6, and Sox9) (Ghazale et al., 2019). In summary, even though differences have been reported, there seems to be significant similarities between rodent and human spinal cord ependymal cells.

While the ependymal cell population contains cells that unlock NSC activity *in vitro* and after SCI, the ependymal layer in mice also encompasses cells that lack stem cell qualities. Two studies have used the GFAP promoter to label a small group of ependymal cells located predominantly in the dorsal ependymal layer, and found that this subpopulation is enriched for primary neurosphere forming capacity. However, these cells do not self-renew efficiently *in vitro*, and they are lost when the neurosphere cultures are passaged (Fiorelli et al., 2013; Sabourin et al., 2009).

The heterogeneity within the ependymal cell population, together with the observation that not all ependymal cells act as stem cells *in vitro* prompted us to investigate the identity of the ependymal cell subpopulation that displays stem cell qualities. The identification and characterization of ependymal neural stem cells is the topic of **Paper III**.

4 CELL-BASED METHODS TO IMPROVE RECOVERY FROM SPINAL CORD INJURY

Studies aimed at repairing the injured spinal cord have focused largely on cell transplantation, and while many cell transplantation studies have shown promising results, transplantation has limitations depending on the source of cells, and is often an invasive procedure (Assinck, Duncan, Hilton, et al., 2017; Barnabé-Heider & Frisé, 2008). Manipulating cell types already present in the injured spinal cord may therefore be an attractive alternative to cell transplantation, although endogenous stem/progenitor cell manipulation in the spinal cord is not as extensively studied, and there is a lot left to learn. This chapter will describe research on different cell-based therapies aimed at improving functional recovery after SCI, and highlight the outstanding gaps in knowledge concerning their efficiency and feasibility.

4.1 CELL TRANSPLANTATION

Researchers have transplanted cells from a wide range of sources to the injured spinal cord. Many studies have shown promising results, including limiting cell death through the release of trophic factors, replacing lost cells, and improving functional recovery (Assinck, Duncan, Hilton, et al., 2017). Some cell types used for such transplantation include mesenchymal stem cells, olfactory ensheathing cells, oligodendrocyte progenitor cells and neural stem and progenitor cells.

Mesenchymal stem cells (MSCs) are tissue stem cells found in, for instance, the adult bone marrow. Transplantation of mesenchymal stem cells to the spinal cord lesion can limit lesion size and exert a neuroprotective and immunomodulatory effect (Assinck, Duncan, Hilton, et al., 2017; Gu et al., 2010), which may result from secreted trophic factors following MSC transplantation (Hawryluk et al., 2012). Given the positive effects attributed to modulating the local lesion microenvironment, as well as the relative ease of harvesting (also autologous) MSCs, this is a widely studied approach to treat SCI. Furthermore, a small number of clinical studies have employed autologous bone marrow mesenchymal stem cell transplantation to treat chronic spinal cord injuries. While these studies have been small or non-randomized, improvement in American Spinal Cord Injury Association (ASIA) Impairment Scale (AIS) scores were observed for some participants (Dai et al., 2013; El-Kheir et al., 2014).

Olfactory ensheathing cells (OECs), which ensheath unmyelinated axons in the olfactory system, have gathered interest as a candidate cell population for cell transplantation to promote functional recovery after SCI. Transplantation of OECs cells can protect neural tissue by releasing neurotrophic factors, and support axonal regeneration and sprouting (Barbour et al., 2013; Takeoka et al., 2011). A few small and non-randomized clinical trials have adopted this approach, with one reporting improved functionality scores after autologous OEC transplantation in a phase I human clinical trial (Tabakow et al., 2013). However, another small clinical trial using OECs from the olfactory mucosa reported considerable variability between the treated patients, and most did not experience significant improvement in AIS scores (Wang et al., 2016). In summary, despite some encouraging

findings, the clinical evidence for OEC transplantation eliciting a functional improvement in humans is still weak (Tabakow et al., 2013; Yao et al., 2018).

OPCs, which produce myelinating cells in the CNS, can be transplanted to the injured spinal cord. OPCs for transplantation can be derived, for instance, from iPS cells or human embryonic stem cells (Kawabata et al., 2016; Keirstead et al., 2005), and there is a growing body of evidence supporting that OPCs transplanted to the injured rodent spinal cord can support remyelination, reduce cavity size in the glial scar, and improve functional recovery (Fu et al., 2018; Kawabata et al., 2016; Keirstead et al., 2005).

Neural stem/progenitor cells transplanted to the spinal cord can promote functional recovery via various means, such as through immunomodulation, by promoting axonal sprouting/regrowth, or by inducing axon remyelination (Assinck, Duncan, Plemel, et al., 2017). NSCs derived from various sources, such as from differentiated embryonic cells and from the adult spinal cord, can produce astrocytes, oligodendrocytes and (typically few) neurons when transplanted into the injured spinal cord of adult rodents (Hofstetter et al., 2005; Yasuda et al., 2011).

Transplantation of *in vitro* expanded NSCs derived from adult rat spinal cord into injured rat spinal cord can facilitate a functional improvement. However, most transplanted NSCs differentiate into astrocytes (Hofstetter et al., 2005). Specifically, while neural stem cells derived from adult rat spinal cord have the capacity to generate neurons as well as astrocytes and oligodendrocytes *in vitro* or when transplanted into a neurogenic region (Shihabuddin et al., 2000), the environment in the spinal cord stimulates glial differentiation. Furthermore, rats that received transplantations suffered from allodynia, a condition characterized by sensations of pain elicited by stimulus that typically does not cause pain. These symptoms may have been a result of the large share of astrocyte differentiation, as stimulating oligodendrocyte differentiation at the expense of astrocyte differentiation by overexpressing *Ngn2* in NSC transplants helped remedy the symptoms (Hofstetter et al., 2005).

Neural stem cells transplanted to the injured spinal cord can restore connectivity across the lesion site. Grafting neural stem cells within a growth factor-enriched fibrin matrix can generate an extensive neuronal differentiation (>25% of transplanted cells), with newly-generated neurons extending long axons into host tissue. These axons are often myelinated by host-derived oligodendrocytes, and the transplantation leads to improvements in locomotion after only a few weeks (Lu et al., 2012). In a follow-up study in rhesus monkeys, neural progenitor cells derived from human embryonic stem cells were transplanted within a growth factor-enriched fibrin matrix to spinal cord lesions. Immunosuppressive treatment was used to manage the immune reaction against the grafted cells. Substantial axonal regrowth across the lesion site was observed after transplantation, which were coupled with functional recovery after 10 weeks and onwards. Transplanting cells with a matrix modulating their local environment can offer enhanced control over the fate of transplanted stem cells, and the results in rhesus monkey suggest that the method can be suitable for clinical translation. (Rosenzweig et al., 2018). A small clinical trial with no control group has shown that NSC

transplantation into human spinal cord lesions can be conducted safely, and while some functional improvement was recorded in two out of four patients, the evidence for functional recovery as a result of NSC transplantation in human is still limited (Curtis et al., 2018).

Cell transplantation therapies may have the potential to rescue structure and function following spinal cord injury. However, the limitations posed by immune reactivity or feasibility of collecting cells for autologous transplantation therapies, as well as concerns regarding safety partially remain. For this reason, it is relevant to explore manipulation of endogenous cells as an alternative to transplantation.

4.2 MANIPULATION OF ENDOGENOUS NEURAL STEM AND PROGENITOR CELLS

Many outstanding questions remain on the topic of manipulating endogenous cells *in vivo* to treat spinal cord injuries. A few investigations have, however, contributed towards some understanding of processes that may be interesting to explore further for modulating the cellular response to spinal cord injury.

Astrocytes are the most abundant glial cells in the central nervous system and given their prevalence, they have been extensively studied to understand what function they offer in the glial scar, by which signals they are controlled, and whether these regulatory processes can be exploited to improve functional recovery after injury. The astrocytic component of the glial scar has both beneficial and detrimental effects on recovery, and targeted manipulations could aim to adapt astrocytic scars to only have beneficial effects on recovery. For example, a therapy may ensure the formation of a barrier that restricts inflammatory cells to the lesion core without blocking axonal regrowth.

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) family of signaling has been implicated as a potential regulator of astrogenesis during development (Bonni et al., 1997). Conditional deletion of a member of the JAK-STAT family, Stat3, in astrocytes highlights that Stat3 is needed for astrocyte hypertrophy and Gfap upregulation, migration, and importantly for the recruitment and organization of the elongated astrocytes that form a scar border between the lesion and the surrounding tissue. Without Stat3 expression in astrocytes, lesions grow larger with an increased spread of inflammation, which ultimately inhibits motor function recovery (Herrmann et al., 2008; Okada et al., 2006; Wanner et al., 2013). On the contrary, conditional ablation of the suppressor of cytokine signaling 3 (Socs3) gene leads to an increased astrocyte migration and an improved barrier function that restricts inflammatory cells to the lesion epicenter. Socs3 ablation also leads to increased scar contraction and improved motor function (Okada et al., 2006).

However, these studies base the assumption that conditional deletion of Stat3 or Socs3 is restricted to just astrocytes on the presence of Gfap or Nestin expression, two genes which are not exclusively expressed in these cells. As a result, the source of the scar-forming astrocytes that are affected by the modulation of Stat3 and Socs3 cannot be determined (Herrmann et al., 2008; Okada et al., 2006; Wanner et al., 2013). They may be stemming

from resident astrocytes or from ependymal neural stem cells (Barnabé-Heider et al., 2010). In fact, the localization in the scar and some of the effects of blocking Stat3 are reminiscent of ependymal-derived astrocytes' behavior after injury. Ependymal cell-derived astrocytes localize to a region close to the lesion core (Barnabé-Heider et al., 2010; Sabelström, Stenudd, & Frisé, 2013), and blocking the ependymal cell contribution to the glial scar leads to worsened secondary damage (Paper I; Sabelström, Stenudd, Réu, et al., 2013).

The ability of ependymal cells to activate stem cell features after injury and generate cells of other lineages pinpoints them as an interesting target for endogenous modulation (Barnabé-Heider et al., 2010; Llorens-Bobadilla et al., 2020; Sabelström, Stenudd, & Frisé, 2013). In spite of this potential, our current knowledge of signals that activate ependymal cells and regulate their fate choices is limited.

As one of the few molecules to have been examined in this regard, β 1-integrin expression is integral in controlling ependymal cells' ability to generate progeny. Without it, ependymal cells are unable to self-renew to maintain their own population while giving rise to migrating progeny (North et al., 2015). Furthermore, without β 1-integrin expression, migrated ependymal progeny expresses Vimentin and Nestin to a lesser extent, while a larger fraction expresses Gfap. These changes were associated with a worsened locomotor recovery after spinal cord injury. The ablation of β 1-integrin also results in decreased expression of Bone Morphogenetic Protein Receptor 1a (BMPR1a), whose signaling typically exerts a beneficial effect on the glial scar by limiting inflammatory infiltration (Sahni et al., 2010). In the SGZ of the hippocampus, BMPR1a signaling regulates proliferation (Mira et al., 2010). These results suggest that β 1-integrin plays a role in ependymal cell self-renewal and fate determination in the glial scar, potentially by modulating BMP signaling.

In **paper II**, we redirected the fate of ependymal neural stem cell progeny by forced expression of the Olig2 transcription factor that is essential for the development of the oligodendrocyte cell lineage (Zhou & Anderson, 2002). While this approach is unlikely to be employed in clinical practice, the study serves as a proof of concept by pinpointing a function of ependymal cells that is attractive to promote, and examining what we may be able to achieve with such a modulation.

Progress has been made towards an understanding of post-injury processes that pose interesting targets for manipulation. In addition, some studies have shown exciting effects from modulating cell populations in the glial scar. However, for an efficient and robust modulation of endogenous cells, a thorough understanding of the identity, roles and regulation of the target cell populations is crucial.

5 PRESENT INVESTIGATION

5.1 AIMS

Paper I – To examine the function of the ependymal neural stem cell response to spinal cord injury

Paper II – To uncover mechanisms underlying lineage potential of ependymal neural stem cell progeny after spinal cord injury, and to redirect their fate after spinal cord injury

Paper III - To narrow down the identity of the endogenous neural stem cells in the spinal cord and examine their activation after injury

5.2 PAPER I

After spinal cord injury, more than half of the newly generated astrocytes in the glial scar are ependymal cell-derived (Barnabé-Heider et al., 2010; Meletis et al., 2008). The effects of the different components of the glial scar remain debated, and both beneficial and detrimental effects on recovery have been attributed to various parts of the scar (Anderson et al., 2016; Bradbury & Burnside, 2019; Dias et al., 2018; Faulkner et al., 2004; Horn et al., 2008; Okada et al., 2006; Silver, 2016; Wanner et al., 2013). In **Paper I**, we examined the function of the scar component generated by ependymal neural stem cells after SCI.

We used a transgenic mouse that let us block the generation of ependymal cell progeny so that they could not be recruited to form the ependymal cell-derived glial scar compartment after injury. We employed a Cre-loxP mouse, where tamoxifen induced recombination specifically in cells expressing FoxJ1 (Meletis et al., 2008), which led to expression of yellow fluorescent protein (YFP) under the Rosa26 locus, as well as to a complete deletion of the *Ras* genes, which are necessary for a cells ability to proliferate and migrate (Drosten et al., 2010).

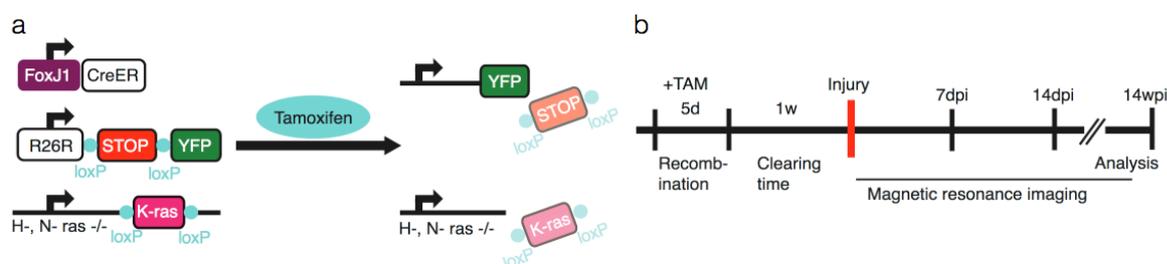


Figure 2 | Inducible labeling and conditional complete deletion of *Ras* genes in ependymal cells.

(A) Schematic description of the strategy used to facilitate induction of YFP labeling and conditional knock-out of the *k-Ras* gene in ependymal cells. (B) Experimental setup. Genetic alterations were induced with tamoxifen. A week later, incision injuries were made. In addition to histological analyses at one week and 14 weeks, some injuries were followed for up to nine weeks using repeated MRI imaging.

Illustrations from Sabelström et al., 2013. Reprinted with permission from the American Association for the Advancement of Science (Science).

Proliferation in recombined ependymal cells was severely reduced in acute spinal cord injuries of mice with complete *Ras* gene knockout. In these mice, almost no progeny was found to have left the central canal one week after injury. Meanwhile, ependymal cells in control mice proliferated vigorously after injury and generated migrating progeny.

In a majority of chronic injuries, we saw a phenotype ranging from mild tissue defects (less compacted scars) to the formation of large cysts at the lesion site. While cyst formation occurs after SCI in most mammals (Ahuja et al., 2017; Bradbury & Burnside, 2019), we do not typically observe them in the injury model we used, and no cysts were formed in the control mice with intact *k-Ras* gene expression.

Furthermore, lesions in mice with a blocked ependymal neural stem cell injury response grew deeper compared to lesions in controls, with more axons in the dorsal corticospinal tract severed in a process secondary to the initial cut. Also, more neurons died in mice without the ependymal neural stem cell injury response (Figure 3). We found that ependymal cells and their progeny express some neurotrophic factors, such as ciliary neurotrophic factor (CNTF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF1), and transforming growth factor beta (TGF β), which may explain how ependymal cells support neuronal survival after SCI (Hawryluk et al., 2012; Kitamura et al., 2007; Tripathi & McTigue, 2008).

It is important to understand the functions and current behavior of biological processes when one wishes to alter them. And, indeed, altering the behavior of ependymal neural stem seems interesting given how our results suggest an important role of their progeny formation in the injury response.

Our results suggest that a complete or near-complete blockade of the ependymal NSC response to SCI is associated with increased secondary damage, which is likely to be associated with a worse functional outcome. However, the question of whether a less severe blockade of ependymal neural stem cells' activity, or a temporally restricted one, could be

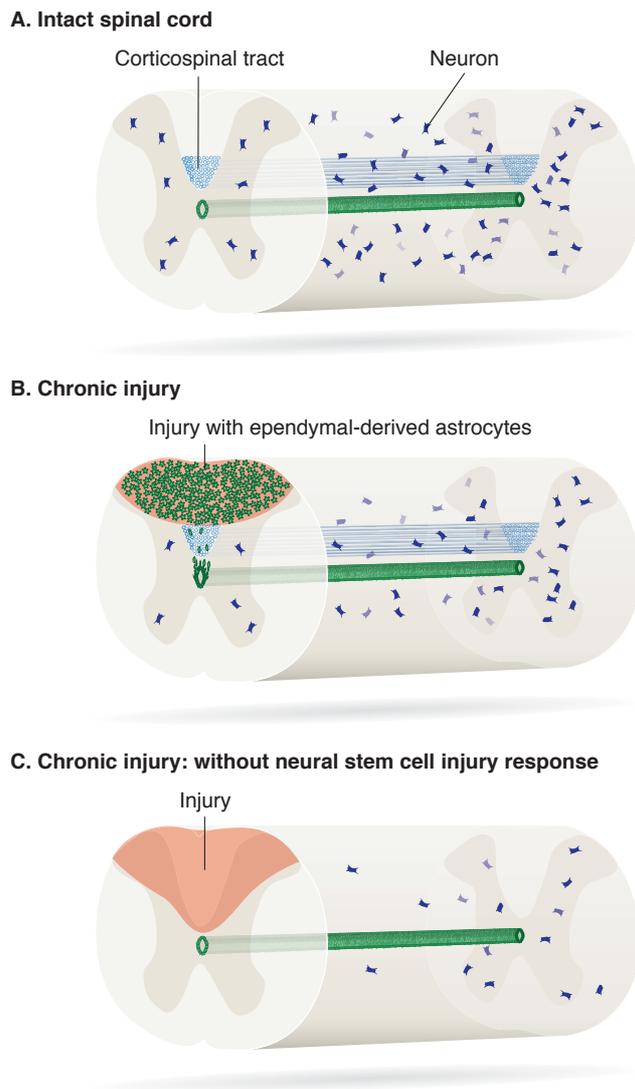


Figure 3 | Long-term consequences of lacking ependymal neural stem cell proliferation after spinal cord injury. (A) An intact spinal cord, showing the ependymal cells residing around the central canal in green. (B) A spinal cord with an incision injury, and an unaltered ependymal cell reaction to the injury. The injury model leaves spared nerve fibers in the corticospinal tract. Ependymal cell progeny produces a scar component that prevents secondary damage of axonal tracts as well as neuronal death. (C) A spinal cord with the same incision injury model, and an ablated ependymal cell response to spinal cord injury. The injury is deeper, additional axonal tracts are severed, and less spared neurons are present at the lesion site.

Illustration from Stenudd et al., 2015. Used with permission from the American Medical Association.

beneficial remains. This has been studied in type A pericytes that give rise to fibroblast-like cells in the scar core. When Dias et al. (2018) moderately reduced the fibrotic scar formation by type A pericytes after SCI, they noted increased axonal sprouting through and beyond the scar, resulting in improved sensorimotor recovery.

Because of the large tissue defects and cysts found in many of the chronic samples from mice without an ependymal cell injury response, the effect on axonal regrowth by ependymal cell-derived scar components could not be examined. If the ependymal cell-generated astrocytic scar component would have a negative effect on axonal regrowth or sprouting, it could be motivated to reduce the production of the astrocytic scar component from ependymal cells.

Some findings of this paper, such as the role of ependymal cells in limiting secondary damage, and their neurotrophic factor expression, have been validated by other researchers, as well as expanded on by examining locomotor function after SCI without an ependymal cell response. The researchers found worsened locomotor recovery after removing the ependymal cells responding to SCI (Cusimano et al., 2018). With these results in mind, it could be interesting to examine whether an overall increase of ependymal cell progeny formation would be beneficial for recovery.

In contrast to the findings of our lab and others, a recent publication found only a minor contribution to scar-forming cells from ependymal progeny (Ren et al., 2017). The authors suggested that the reason for the large discrepancy between our findings and theirs, may be our use of the dorsal funiculus incision injury model, which in theory may directly harm ependymal cells. In this article, we use a superficial incision injury, where we see spared nerve fibers in the corticospinal tract of control mice, implying that we could not have severed the ependymal layer directly. We also see a significant reaction by ependymal cells after contusion and crush injuries in paper II and III. Furthermore, the authors raised concern about the fidelity of the human *Foxj1* promoter that we use (Meletis et al., 2008). We have confirmed that *Foxj1*-CreER^{T2} recombined ependymal cells (and *Troy*-CreER^{T2} recombined ependymal cells that were studied in Paper III) express *FoxJ1* at the mRNA and protein levels. Moreover, in paper III, we traced the progeny of EpA ependymal cells using a non-*Foxj1* dependent CreER^{T2} mouse line (*Troy*-CreER^{T2}). A recent study suggests that *Foxj1* is important for the ependymal cell reaction to spinal cord injury (Li et al., 2018). The loss of *Foxj1* expression on one allele in the *FoxJ1*-CreER knock-in line used by Ren et al. (Muthusamy et al., 2014) may explain their finding of a minimal injury reaction.

In summary, Paper I ties functions including limiting secondary growth of spinal cord lesions and limiting neuronal death to ependymal cells and ependymal cell-derived astrocytes in the glial scar. The study sheds some light on the roles of a subtype of cells in the astrocyte component in the glial scar, and shows us that ependymal cells can constitute an interesting target for therapeutic modulation of an endogenous cell population to treat spinal cord injury.

5.3 PAPER II

Ependymal cells in the spinal cord respond to injury by dividing rapidly and generating astrocytes in the glial scar as well oligodendrocytes, although the contribution to the oligodendrocyte lineage is minimal (Barnabé-Heider et al., 2010). Increasing the supply of oligodendrocytes via cell transplantation improves remyelination and functional recovery after spinal cord injury (Kawabata et al., 2016; Salewski et al., 2015; Yasuda et al., 2011). For that reason, we set out to explore if promoting an oligodendrocyte fate choice from endogenous cells could improve outcome after spinal cord injury.

In **paper II**, we used single cell ATAC-seq data (Satpathy et al., 2019) integrated with single cell RNA-seq data (from Zeisel et al., 2018) to assess the lineage potential of ependymal cells and other scar-forming populations in the spinal cord by integrating their gene expression and chromatin accessibility patterns (Figure 4).

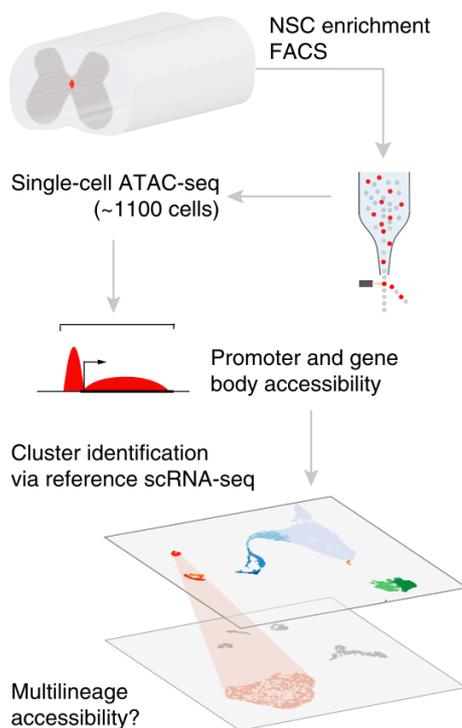


Figure 4 | Illustration of single cell chromatin accessibility experiment. Fluorescence activated cell sorting (FACS) was used to obtain a sample of spinal cord cells enriched for recombined tdTomato expressing ependymal cells. The collected sample was used for single cell ATAC-seq, which was combined with RNA-seq data to study chromatin accessibility in cells from different populations in the mouse spinal cord.

Illustration from Llorens-Bobadilla et al. (2020). Used with permission from American Association for the Advancement of Science (Science).

To this end, we sorted cells extracted from the uninjured and injured spinal cord of mice with an induced expression of tdTomato under the Foxj1-CreER^{T2} promoter (Foxj1-tdT in Figure 5). We collected a population of cells enriched for, but not always expressing, tdTomato – meaning we collected both recombined ependymal cells and other cell types present in the intact and injured spinal cord. In the integrated ATAC-seq and RNA-seq data sets, we found clusters containing astrocytes, oligodendrocyte lineage cells, vascular endothelial cells, pericytes, and ependymal cells. Looking at the ATAC-seq data of the different clusters, we found that Olig2 and Sox10 binding sites were accessible not only in oligodendrocyte lineage cells, as expected, but also in ependymal cells. While both Olig2 and Sox10 are expressed by oligodendrocyte lineage cells, neither is expressed by ependymal cells.

Finding chromatin accessibility patterns showing permissiveness for oligodendrogenesis programs prompted us to ask whether we could activate oligodendrogenesis by activating the expression of the Olig2 gene. To this end, we generated a transgenic mouse line allowing for simultaneous induction of Olig2 expression and tdTomato reporter expression specifically in FoxJ1-expressing ependymal cells (Foxj1-Olig2-tdT in Figure 5).

We made spinal cord injuries in Foxj1-Olig2-tdT mice and Foxj1-tdT control mice. Using a combination of RNA-seq and ATAC-seq, we examined recombined cells in uninjured spinal cords, as well as 1 and 5 days after injury. We found increased chromatin accessibility of the Olig2 binding sites in cells from Foxj1-Olig2-tdT mice. Furthermore, we found that Sox10 enhancer regions gained chromatin accessibility, which was paired with an increased Sox10 expression after SCI. This implies that we were able to activate an oligodendrogenesis program by introducing Olig2 expression and a SCI.

After establishing that we could turn on an oligodendrogenesis program by inducing Olig2 expression in ependymal cells, we asked how that would affect their injury response. We analyzed injuries of Foxj1-Olig2-tdT mice and controls 2, 4 and 12 weeks after injury, focusing on oligodendrogenesis. We found a sizeable net addition of Sox10 expressing oligodendrocyte lineage cells at all time points studied, looking both at single cell RNA sequencing data and protein expression in tissue. On average, ~32,000 recombined oligodendrocyte lineage cells were found in a lesion 12 weeks after injury. This is comparable to the amounts observed after NSC transplantation (Yasuda et al., 2011). In the single cell RNA-seq data, we found that the oligodendrogenesis that we induced from ependymal cells seemed to recapitulate developmental oligodendrogenesis (for instance, we found PDGFR α expression at early time points, and Myrf expression at late time points) (Steven A. Goldman & Kuypers, 2015).

To examine if we could introduce the same oligodendrogenesis program in astrocytes, we generated Connexin 30 (Cx30)-Olig2-tdT mice to label and introduce a forced Olig2 expression in astrocytes. When looking at injuries from Cx30-Olig2-tdT mice, we did not find the efficient generation of Olig2-expressing cells that we observed from ependymal cells.

Ependymal cells:

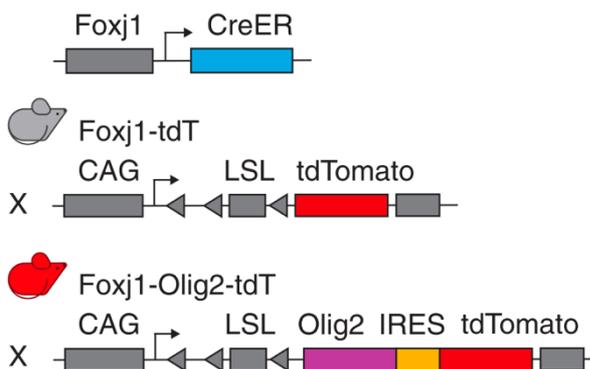


Figure 5 | Schematic description of the strategy used to induce labeling and Olig2 expression in ependymal cells. FoxJ1-tdT denotes the control mice (without Olig2 expression), while Foxj1-Olig2-tdT shows the design of the construct turning on Olig2 expression with the labeling of Foxj1⁺ ependymal cells.

Illustration from Llorens-Bobadilla et al. (2020). Used with permission from American Association for the Advancement of Science (AAAS).

This supports that ependymal cells are specifically permissive to oligodendrogenesis, and highlights ependymal cells as an interesting target for stimulating oligodendrogenesis from endogenous cells.

At the latest time point after injury in Foxj1-Olig2-tdT mice, we confirmed the presence of myelinating oligodendrocytes generated from ependymal cells with multiple methods. First, we found co-localization of tdTomato with myelin basic protein (MBP) that marks myelinating oligodendrocytes. Next, we used expansion microscopy on Foxj1-Olig2-tdT mice crossed with Thy1-GFP which marks neurofilaments with Green Fluorescent Protein (GFP), and visualized axons wrapped by tdTomato expressing ependymal-derived oligodendrocytes. Lastly, we confirmed the findings using electron microscopy.

Using a contusion injury model, we examined if the increased recruitment to the oligodendrocyte lineage had an effect on locomotion. We did not detect any difference in locomotion measured by Basso Mouse Scale (BMS) or hindlimb scores during 12 weeks after injury (Basso et al., 2006). To further understand the potential for recovery of axonal conductance induced by activation of the latent oligodendrogenesis program in ependymal cells, we performed parallel electrophysiology and optogenetics *in vivo*. We injected adeno associated virus (AAV)-CAG-ChR2-EGFP into the motor cortex in order to label cortical neurons and their axons in the corticospinal tract. Optical stimulation was applied rostral to the lesion, and recordings were made rostral and caudal to the lesion. We found a higher conduction velocity rostral to injuries in Foxj1-Olig2-tdT mice compared to in controls with unaltered Olig2 expression, and the conduction velocity correlated with the share of myelinated axons in the corticospinal tract.

The improved axonal conductance observed in Foxj1-Olig2-tdT showed that the oligodendrogenesis we induced resulted in a measurable improvement through myelination of axons. Despite the large net contribution to the oligodendrocyte lineage observed in tissue, we did not see an effect on gross locomotion. This is in line with the literature, since it has been reported that manipulation of myelin alone does not affect locomotion (Duncan et al., 2018).

In this study we identified ependymal neural stem cells as an interesting target population for stimulating endogenous oligodendrogenesis. This could prove an attractive option for regenerative treatments, although a greater understanding of the signaling pathways that control the process is needed.

5.4 PAPER III

All spinal cord ependymal cells are not the same. The spinal cord ependymal cell population contains cells of different shapes, and cells with different protein expression profiles (Bruni & Reddy, 1987; Garcia-Ovejero et al., 2013; Meletis et al., 2008; Sabourin et al., 2009).

Previous work has shown that the ependymal layer encompasses cells that cannot self-renew efficiently throughout passages *in vitro*, while it is known that the ependymal cell population contains cells that self-renew efficiently and have stem cell capacities *in vitro* (Barnabé-Heider et al., 2010; Fiorelli et al., 2013; Meletis et al., 2008; Sabourin et al., 2009). This prompted us to address the question of which population, among the ependymal cells, that shows stem cell abilities.

Troy (Tnfrsf19) is a Wnt target gene that is present in stem cell populations of the gastrointestinal tract as well as the SVZ of the brain (Basak et al., 2018; Faflek et al., 2013; Stange et al., 2013). We examined the stem cell capacities of Troy-expressing cells using a Troy-CreER^{T2} mouse line on a Rosa26-tdTomato background.

Only a minority of all spinal cord ependymal cells (~8%) were recombined in the Troy-CreER^{T2} mouse line, and we refer to those as EpA cells. EpA cells are dispersed throughout the ependymal layer, although they are more frequently found dorsally.

We cultured cells from dissociated spinal cords of recombined Troy-CreER^{T2} mice, and found that the primary neurosphere cultures were not enriched for neurospheres generated by recombined EpA cells. However, over the passages the recombination rate increased until a vast majority of neurospheres originated from the EpA cell population at p2 and onwards. Since recombination is seldom complete, this implies that all or nearly all of the cells with *in vitro* NSC capacity in the spinal cord resides in the EpA ependymal cell subpopulation.

Next, we examined the response of EpA ependymal cells to injury. After SCI, these cells started dividing and generated migrating progeny that differentiated into astrocytes and oligodendrocytes without the EpA ependymal cell population being depleted in the process. This implies that EpA cells are able to self-renew while they generate differentiating progeny, although it was not clear from this experiment whether the same cells were able to both self-renew and generate migrating progeny.

To better understand the dynamics of the EpA cell reaction to SCI, we crossed the Troy-CreER^{T2} mouse line with a Rosa26-Confetti reporter mouse line (Figure 6A). We induced recombination to label EpA cells. Recombination led to the expression of any of the four fluorescent reporters CFP, GFP, RFP or YFP. The mice were subjected to injury two weeks after tamoxifen treatment, and the injured spinal cords were analyzed after two additional weeks (Figure 6B). The recombination in the injury sites was low, with an average of less than 3 EpA cells per fluorescent reporter in a whole injury site (spanning 1.1 mm, on average). The low recombination rate in combination with the stochastic expression of fluorescent proteins enabled us to identify and analyze single migrating clones.

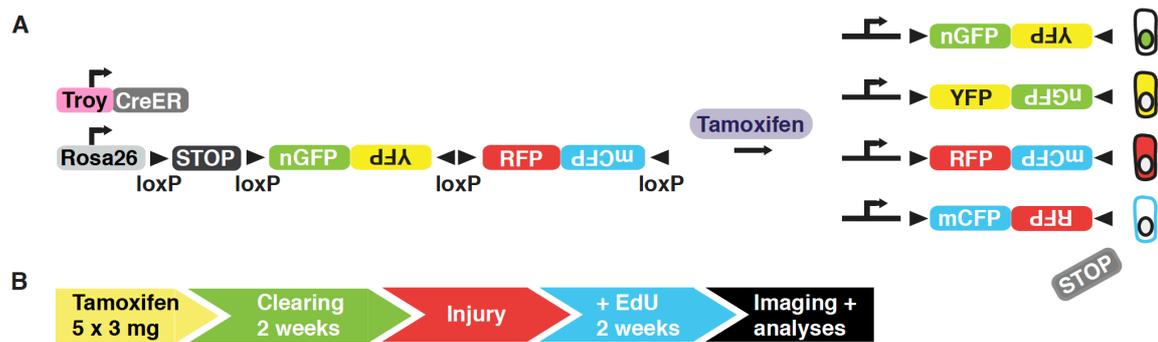


Figure 6 | Single cell clonal analysis with Troy-CreER^{T2} on a reporter background. (A) Schematic description of the strategy to induce stochastic labeling of EpA cells. (B) Experimental timeline. Two weeks after recombination to label EpA cells, dorsal funiculus injuries were made. The injuries were analyzed 2 weeks later.

We found 20 clones containing migrating EpA progeny in 12 spinal cords. Each clone contained between 1 and 6 migrating cells, and they spread more in the dorso-lateral direction than in the rostro-caudal direction. For the vast majority of clones containing migrating progeny, we could identify an EpA ependymal founder cell. This suggested to us that EpA cells are able to generate migrating progeny without being consumed in the process, meaning they show self-renewal on a single cell level. Among the recombined cells, we found evidence for different modes of division. An ependymal cell could give rise to (i) only ependymal cells, (ii) ependymal cell(s) + astrocyte(s), and (iii) only astrocytes. In 5 clones with mixed progeny (ii), we found only one single ependymal cell and one single migrated cell. In those cases, it seems likely that the founding ependymal cell had undergone a single asymmetric division.

Owing to the small ependymal cell contribution towards the oligodendrocyte lineage in combination with the early time point at which we conducted the *in vivo* clonal analysis, we did not identify any clone containing oligodendrocyte lineage. Therefore, the question of fate restriction in the EpA neural stem cell population remains unanswered. In the SVZ niche, there is a functional heterogeneity, and the NSCs that generate oligodendrocytes are typically not the same NSCs as those who generate astrocytes and neurons (Ortega et al., 2013).

To understand the molecular underpinnings of the process leading up to the injury reaction of EpA cells, we conducted single cell RNA sequencing on EpA cells from uninjured spinal cord, and from spinal cord lesions 3 days after injury – a time point characterized by the initial recruitment of ependymal cell progeny, which begin migration from the central canal to form astrocytes in the glial scar and a few oligodendrocytes (Sabelström, Stenudd, & Frisén, 2013).

In the scRNAseq data set, we identified three clusters containing ependymal cells, which we analyzed further. One cluster, EpA1, contained cells from both uninjured and injured spinal cord. These appeared as highly differentiated ependymal cells, and expressed high levels of ependymal cell related genes, such as Foxj1, Fam183b, and Rarres2. Another cluster, EpA2, encompassed cells from injured spinal cords, with high expression of ribosomal RNA as well

as translation related genes. Lastly, EpA3 contained proliferating cells from injured spinal cords. In both EpA2 and EpA3 clusters, ependymal cell markers were downregulated. Conversely, genes implicated in differentiation, cell migration and proliferation were upregulated. Cells in EpA2 and EpA3 also had increased signaling entropy compared to EpA1, which implies a greater multilineage potential, as well as a greater similarity to SVZ NSCs (EpA2) and transit amplifying cells (EpA3).

In summary, we observed highly differentiated EpA ependymal cells downregulate ependymal cell markers shortly after SCI. Instead, the EpA cells upregulated genes that suggest a transition towards a more stem cell-like state after injury. They expressed more ribosomal genes, as well other protein synthesis related genes, which is reminiscent of the changes in mRNA expression observed when quiescent NSCs in the SVZ are activated (Basak et al., 2018; Llorens-Bobadilla et al., 2015).

The process in which EpA ependymal cells revert from a highly differentiated cell to one with stem cell qualities carries similarity to that of local astrocytes in the striatum transitioning to a stem cell-state after striatal stroke or Notch signaling depletion (J. Magnusson et al., 2014; J. P. Magnusson et al., 2020).

In the EpA ependymal cell subpopulation, we have identified a small group of ependymal cells that harbor much, if not all, of the *in vitro* stem cell capacity of the adult spinal cord. They behave distinctly different from two previously described ependymal cell subgroups (Fiorelli et al., 2013; Sabourin et al., 2009), which both expand *in vitro* but lack ability to self-renew over time.

6 CONCLUSIONS AND PERSPECTIVES

This section will focus on our conclusions from the work presented in this thesis, and future perspectives relating to the thesis work. Specifically, we found that:

1. Ependymal cells can limit secondary tissue damage and reduce neuronal loss after SCI
2. We can activate a latent oligodendrogenesis program in ependymal cells, and the newly formed oligodendrocytes myelinate nerves and restore conductance after SCI
3. The EpA ependymal cell subtype harbors nearly all *in vitro* stem cell potential in the adult spinal cord. Upon injury, highly differentiated EpA ependymal cells revert to a stem cell-like state.

The astrocytic component of the glial scar has both beneficial and detrimental effects on the pathological processes after SCI. In paper I, we showed that the ependymal cell response to SCI is beneficial for limiting lesion size and neuronal loss. Similarly, work by other research groups have shown that different parts of the astrocytic scar play different roles in recovery (Herrmann et al., 2008; Okada et al., 2006; Wanner et al., 2013). However, the source of the astrocytes manipulated in these studies is unknown since they used genes present in both ependymal progeny and astrocytes to drive the transgene expression. An increased understanding of whether the heterogeneity observed within the glial scar is tied to lineage or to location in the scar will be important to devise effective and specific treatments targeting the intended part of the glial scar. An inducible and targeted reduction of the scar component from astrocytes (for instance crossing a CreER^{T2} line that targets resident astrocytes, Cx30-CreER^{T2}, with a Rasless mouse line to block their proliferation) could bring clarity on the contribution of different scar-forming populations.

In paper I, we learned that the physiological amount of progeny formed by ependymal cells after injury renders a more favorable outcome compared to when we blocked the generation of the ependymal-derived scar component. However, this experiment did not answer the question of whether the outcome would be better if we had an even larger contribution of ependymal-derived astrocytes to the scar. In paper II, we generated more ependymal-derived astrocytes in mice with forced Olig2 expression compared to controls. Unfortunately, it is difficult to draw any conclusion on the effects on this increase in astrocyte numbers, since the addition of new oligodendrocyte lineage cells is far greater. Finding the amount of ependymal-derived astrocytes and other scar cell types that is associated with the most favorable outcome will be useful to learn what we can and want to achieve when manipulating scar-forming cell populations.

In paper III, we showed that most of (if not all) the *in vitro* NSC capacity of the adult spinal cord is harbored by the EpA ependymal cell subpopulation. An open question is how large a share of the *in vivo* response they account for. To improve our understanding of the EpA ependymal cells relative contribution to the ependymal cell response to spinal cord injury, an important aspect to understand would be the long-term functional role of the EpA injury

reaction. Examining the long-term consequences of selectively blocking the injury response by EpA ependymal cells with a similar methodology as we employed in Paper I could provide an answer to this question.

In paper III, we found that ependymal cells are activated upon injury to attain a more stem cell-like state. An understanding of the signals that initiate the cascade of EpA cell activation upon injury will be essential to control their activation. With the importance of Stat3 for the recruitment of a scar component residing where ependymal cell progeny resides in the scar, the JAK/STAT pathway is an interesting potential target to examine.

We identified a latent oligodendrogenesis program in ependymal cells, and activated it by forced Olig2 expression combined with SCI. However, no oligodendrocyte gene expression was observed without an injury, not even a year after Olig2 induction. This is intriguing in the context of Paper III, where we see that EpA ependymal cells revert to a stem cell-like state with a larger multipotent potential within days after injury. Perhaps this process is required to allow the fate switch of ependymal progeny induced in Paper II.

While increasing oligodendrogenesis by ependymal cells appears attractive, the method employed to activate the oligodendrogenesis program in ependymal cells in paper II is an unlikely candidate for translation to human application. To translate these findings and circumvent the risks associated with the overexpression of a transcription factor, it will be important to address outstanding questions regarding the signaling pathways that regulate the fate choices of ependymal cell progeny.

To assess the feasibility of translating the results in Paper II and other findings from modulating stem cell behaviors to human use, it will be important to learn more about the differences between mouse and human ependymal cells. To this end, repeating the experiment with combined RNA-seq and ATAC-seq employed in Paper II on human spinal cord cells could give an interesting perspective on the similarities between ependymal cells in humans and mice.

Through the work behind Paper II, we learned that we can manipulate the fate of spinal cord ependymal cell progeny towards the oligodendrocyte lineage to produce >30,000 new oligodendrocyte lineage cells after SCI.

NSC transplantation can improve locomotor function after spinal cord injury, and it has been reported that the effect requires the development of myelinating oligodendrocytes (Yasuda et al., 2011). In contrast, a recent study showed that locomotor recovery after SCI is not dependent on remyelination (Duncan et al., 2018). This is in line with our results, as we did not measure any improvement in locomotor recovery.

In a recent study, the authors stimulated axonal regrowth through spinal cord lesions using a growth factor cocktail. They showed more than a 100-fold increase in axonal regrowth, but failed to show any improvement in locomotion (Anderson et al., 2018). Newly regenerated axons are frequently not myelinated (Alto et al., 2009), and improving their conduction

velocity can promote functional recovery (Bei et al., 2017). Together, these results point towards a need for combinatorial treatment, where multiple processes are modulated together, for instance by promoting axonal regrowth and oligodendrogenesis from endogenous neural stem cells in parallel.

With the research presented in this thesis, we have laid some groundwork that is necessary in order to utilize endogenous stem cells for treating spinal cord injury. We have learnt about the functions of the ependymal cell response to spinal cord injury, about the plasticity within the ependymal cell population, and about the identity of ependymal cells harboring stem cell potential, as well as their activation after injury. We have also discussed some challenges ahead, and reaching the end of this doctoral thesis, I am excited about the discoveries that are still ahead of us.

7 ACKNOWLEDGEMENTS

First of all, I want to thank my advisor **Jonas** for giving me the opportunity to work on these exciting projects, and for being a supportive person in my life. You've put together a fantastic lab.

My co-advisor **Fanie**, you've been a great support. I will not forget our long debates on experiments in your office!

Thanks to past and current members of the **Frisén lab** for being great colleagues. **Hanna**, I am so grateful to you for introducing me to the lab and to endymal cells. Thank you for your patience and your companionship during many night shifts in the lab! **James** and **Enric**, you've been fantastic endymal cell buddies. Thank you for always being generous with your knowledge and your time. **Johanna**, you've been a great colleague and friend. It's been fun to share your excitement about discoveries in the lab and I really enjoyed (y)our tumor experiment. **Joanna**, I'm happy you hustled your way into the lab with me! I've had fun sharing office and cute cat pictures with you. **Jeff**, thanks for sharing a perfect mix of insights about life, research and funny YouTube clips with me over the years. I've enjoyed sharing the office with you. **Marta**, Thanks for always being a helpful colleague, and for all the Italian games! **Embla**, I'm happy we met in the lab. You've been a great colleague, and I valued your advice on clinical medicine. Last but not least – thanks for arranging fantastic lab parties! **Ilke**, I've enjoyed sharing the office with you. I already miss overhearing fun conversations from your desk! **Jens**, you've been a great colleague. You made me feel really welcome my first day in the lab by taking initiative and asking me questions about research. **Giuseppe**, thanks for always looking out for people around you. **Margherita**, thank you for your solid stats advice, your generosity and for awesome company! **Ionut**, thanks for lightening up the mood during lunches with your sense of humor. **Mathew**, wow, I'm glad for your sake I did not find out how awesome you are at proofreading before you went through chapter 4! Thanks for that and for being a genuinely supportive colleague. **Kanar, Sofia, Aleksandra, Aurelie, Maggie, Mehdi, Hagen and Klas**, Thanks for making the lab a great place to work in. I've really enjoyed our chats over food, beers and lab work. **Pedro**, I miss your window drawing, excitement about new ideas and podcast recitals! **Camilla**, you're such a great addition to the lab. Always generous, including and ready to contribute. **Michael**, never stop telling your awesome rat stories. **Leonie**, I'm excited about you joining the spinal cord crew and inheriting my old cords! You'll do some awesome science, and I'm looking forward to read about it. ☺ **Sue**, I'm happy you joined us and excited about all the cool endymal cell research you've got ahead of you! **CJ**, you've been a kind and fun nerdy desk buddy and you'll be missed! I'm going to have a lot of time to practice for our next type race now. **Julia**, thanks for friendly company and chats in the lab! **Marcelo** and **Sarantis**, thanks for fantastic help and patience during cell sorts! **Helena**, you're completely *irreplaceable*! Thank you for great conversations and fantastic support over the years.

To all the students who have worked with us – thanks for making the lab a fantastic place to work in. To the students I supervised in the lab: **Rebecka, Shupe, Melanie, Chantal,** and **Antonio,** thank you – I’ve had so much fun teaching you, learning from you, and learning with you.

Fanie Barnabé-Heider lab. **Xiaofei,** you’re a generous colleague. It’s been great to follow each other as we progressed throughout our PhD journeys. **Elisa,** you’ve been a great colleague. I wish we would’ve had the chance to work more together. **Nicolas** and **Konstantinos** – thanks for friendly chats and collaboration.

Christian Göritz lab. Thank you for collaboration and advice. **Christian,** I enjoyed having my articles scrutinized by you. You have a great eye for detail. **David,** I admire your diligence, you’re a great scientist. Thanks for all your support, for generosity with your knowledge and for great company during the night shifts in the lab! **Daniel,** you’ve been such a generous and helpful colleague! **Eduardo, Soniya, Benjamin,** and **Jannis** – thanks for always being friendly and helpful colleagues! Enjoyed your company during long experiments in KMW/KMB.

András Simon lab. Thank you, I really enjoyed sharing an office with you before the move to Biomedicum – I’ve missed it since even if you were only 10 meters away! **Shahuls** and **Anoops** banter. :) Rum and chitchat with Anoop! **Heng’s** fantastic and perfectly timed sarcastic comments after long periods of silence. I have enjoyed sharing an office and, on occasion, insights with all of you; **Daniel, Gonzalo, Laure, Iv, Tiago, Ahmed, Alberto, Zeyu, Matthew,** and **András.**

Olaf Bergman lab. Thank you – **Olaf,** I already miss your awesome dance moves. **Enikő** and **Marion,** I’m happy to have shared lab with you both, and thankful for nice conversations over cakes!

Rickard Sandberg lab. It’s been great ~sharing the lab space over the last few years. ☺ **Anton,** I’ve enjoyed your addition to the Frisén lunch crew.

Jean Hausser lab. It’s been great meeting some of you over lunch, and I’ve enjoyed the whiteboard.

David Grommisch, thanks for being a generous and fun colleague to do Troy experiments with, and a special thanks for excited teaching on how to find(!) and roll up a gut.

Our collaborators, **Peter Conner** and **Sahar Aski,** I had a fantastic time learning about MRI machines and precision mouse anesthesia from you! **Hans Blom** and **Hjalmar Brismar,** thank you for extraordinary support on super-resolution microscopy.

Mattias Karlén, thank you for fantastic illustrations!

Matti Nikkola, thank you for always being on the students’ side. You’ve been a great support and I have appreciated our conversations about knowledge, teaching and life.

To **all my new colleagues at Grace Health!** You have made me feel so welcome, and I am so grateful that we found each other. ☺

I want to thank **Emil and K**, my two dearest medical school friends. Emil, without you I wouldn't have found my way to class. ☺ Who knows where I'd be today? K, I value the time we spent discussing how to improve often completely unimportant aspects of the world around us!

Bella, I'm so thankful that we crossed paths in the lab! You've become an important friend to me, and while we've both had a lot going on in our lives in periods – I know I can always count on you.

Mamma, thank you for your warmth and your never-ending support. **Pappa**, thank you for always helping me trust my own abilities. To my sister **Lina** and my brother **Martin**, thank you – every time we meet I feel like we saw each other yesterday.

Francesco, thank you for your love, for all the invaluable time we've spent together, and for your unconditional support and encouragement. And thank you for the fantastic songs. I will always be there for you.

Ada, thank you for always keeping me in the moment with your energy, your crazy ideas and your fantastic laughter.

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