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# THE DEVELOPMENT AND POTENTIAL OF SPINAL CORD STEM AND PROGENITOR CELLS

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# THE DEVELOPMENT AND POTENTIAL OF SPINAL CORD STEM AND PROGENITOR CELLS

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Dedicated to my beloved mother, father and my grandmother

*致我的家人*

# POPULAR SCIENCE SUMMARY

Spinal cord controls the communication between the brain and the body and coordinates the motor movements and sensory functions. When an injury occurs to the spinal cord, the signal bridge between the brain and the body is impaired, resulting in partly or fully functional loss of the body below the lesion level. The main reason is that after spinal cord injury (SCI), the neurons and other helper cells in the spinal cord die, and this cell loss is a continuous process even long after the injury has occurred. There is no cure so far for SCI, but several new treatments based on stem cell therapy have been studied in this thesis.

Stem cells are cells that can generate more identical cells to themselves, and can also become other cell types as "self-reproductive transformers". After SCI, spinal cord stem cells can activate the self-production system to produce more stem cells, move to the lesion area, and also become other helper cells to compensate for the cell loss. However, we found that the capacity of spinal cord stem cells decreases dramatically during aging, and the ways they contribute to self-recovery are different at young and mature age, suggesting that the current standard treatments for SCI need to take age into more consideration. Moreover, the regeneration capacity of stem cells and other helper cells at adult age has been largely determined during embryonic development, but many current studies only focus on the adult stage and overlook the link between development and regeneration. Here we found a gene, called FoxJ1, which regulates the embryonic development of the spinal cord, and is essential to maintain the stem cell potential in adulthood and after SCI. Targeting this gene could be a novel approach to recruit more spinal cord stem cells and provide better SCI treatments.

Furthermore, a severe SCI patient received the transplantation of cells extracted from part of his own olfactory system in the brain, which made him the first severe SCI patient walk again with limited side effects. The surgery was successful, but the reasons were unknown, leading to a difficulty in achieving the same effect on other SCI patients. We studied the mechanisms behind and found that this type of cells originally from the olfactory system cannot generate other cells after being transplanted into the injured spinal cord, but they can stimulate the spinal cord stem cells to generate more stem cells and give rise to a more substantial number of beneficial helper cells. Moreover, it is widely believed that there are no newborn neurons in the adult spinal cord, resulting in the permanent loss of neurons in case of SCI. However, our study found that after this cell therapy, there are newborn neurons in the spinal cord after SCI. We suggest that a further study on the molecules produced by this cell therapy will be of high importance for developing new cell therapies for SCI.

Altogether, this thesis sheds some light on the understandings about how the spinal cord is developed, and how this developmental process influences the stem cell potential at adulthood and after SCI. This thesis provides some inspirations to target specific cell types and genes for new therapeutic possibilities for SCI.

作为中枢神经系统的一部分，脊髓控制着运动和感觉，是大脑和躯体的沟通的桥梁。如果脊髓发生损伤，大脑和身体之间的信号传输就会受到阻碍，导致在损伤位置以下甚至全部躯体功能的丧失。这主要是由于损伤发生后，脊髓中的神经元和其他的辅助细胞大量死亡，而且细胞死亡过程会一直持续到损伤发生后的很长一段时间。目前脊髓损伤尚无法治愈，但本文研究了几种基于干细胞的新疗法。

干细胞是一种能自我更新，并可以分化成其他细胞类型的“变形金刚”。脊髓损伤后，脊髓干细胞能够激活自我更新的能力，产生更多的干细胞，并且能迁移到病变区域，分化成其他类型的细胞以补偿损伤造成的细胞死亡。然而，我们发现在机体衰老的过程中，脊髓干细胞的这种自我更新和变化能力急剧下降。因此在年轻和年老时期，脊髓的自我恢复的方式大不相同，提示当前脊髓损伤的治疗方案需要更进一步考虑年龄的影响。此外，成年期干细胞和其他辅助细胞的再生能力在胚胎发育过程中已基本被确定，但目前许多研究却仅关注成体期，忽略了胚胎发育和组织再生的关联。本论文中，我们发现了一种叫 FoxJ1 的基因，它能调节脊髓的胚胎发育，并且在成年期，当脊髓发生损伤后，对维持干细胞的潜能至关重要。靶向这个基因可能会带来新的基因疗法，刺激并获得更多的脊髓干细胞，从而提供更好的脊髓损伤治疗的可能性。

此外，一名严重脊髓损伤患者接受了细胞移植手术，细胞来源于他自己的嗅觉系统。这个手术使他成为第一个能重新获得行走能力的严重脊髓损伤患者，而且术后副作用很小。但由于机理不明，这个手术的成功并不能使其他脊髓损伤的患者获得同样的疗效。我们对这种疗法进行了研究，发现虽然这种类型的细胞在移植到受损的脊髓后并不能产生其他细胞，但是它们可以刺激脊髓干细胞产生更多的干细胞并分化出大量有益的辅助细胞。更有趣的是，人们普遍认为，成年脊髓中不能再出生出新的神经元，因此脊髓损伤导致的神经元丧失是永久的。然而，我们的研究发现，在经过这种细胞疗法之后，损伤的脊髓出现了新生的神经元。因此，我们建议进一步研究究竟是这种细胞产生的何种分子起的作用，这对于促进脊髓损伤的新细胞疗法的发现非常重要。

总之，本文对研究脊髓发育以及脊髓发育过程如何影响成年和脊髓损伤后的干细胞潜能提供了新的思路，并为脊髓损伤的细胞和基因疗法提供了一些新启示，从而为脊髓损伤的治疗提供了新的可能性。

# ABSTRACT

Spinal cord injury (SCI) is an incurable condition, which is mainly due to the highly limited regenerative potential of the adult spinal cord. The discovery of ependymal cells as the source of spinal cord stem cells raises hopes for the development of new therapies, while cell transplantations for SCI also provide promising means for potential treatments. However, curing SCI has been proven difficult as the potential of these progenitors and ependymal stem cells is still understudied. Moreover, the development of the spinal cord is a key factor influencing the regenerative potential of neural stem/progenitor stem cells, but the link between the development of spinal cord progenitors and adult spinal cord regeneration has been largely overlooked. By using different transgenic mouse lines and biomedical techniques, we studied the neural progenitors and stem cells during spinal cord development, after SCI and after cell transplantation in this thesis.

FoxJ1 is traditionally regarded as a transcription factor involved in ciliogenesis and a specific marker for ependymal cells. In **Paper I**, however, we discovered that FoxJ1 is transiently expressed in neuronal and glial progenitors, which will further give rise to subsets of interneurons, two subsets of astrocytes and all ependymal cells. FoxJ1 is required for the maintenance of stemness of the progenitors during development and the stem cell potential during adulthood. After SCI, FoxJ1 is required for the normal stem cell potential, proliferation and migration of ependymal cells to promote regeneration.

After the early developmental stage, in **Paper II**, we observed that the stem cell potential is fully confined to ependymal cells from P10 in mice, and the potential of self-renewal and oligodendrocytic differentiation decreases over time. Juvenile ependymal cells have higher stem cell potential after SCI than adult ones, but their contribution to the glial scar formation *in vivo* is lesion size- and age-dependent. We found that the resident astrocytes and stromal derived pericytes show higher regenerative potential at the juvenile stage, and ependymal cells serve as a backup regeneration candidate after SCI.

Clinically in the adult spinal cord, the transplantation of bulbar olfactory ensheathing cell (bOEC) has shown significant functional recovery in SCI patients, but the mechanisms are not elucidated. In **Paper III**, we found that after SCI, bOEC transplantation increases the proliferation and self-renewal potential of ependymal cells. The transplantation of bOECs promotes higher astrocytic differentiation of ependymal cells but reduces the axonal growth inhibitors after SCI. The microenvironment of the injured spinal cord is enriched after bOEC transplantation in terms of less axonal growth inhibitor, a higher level of neurotrophic factors and better neuronal survival. Unexpectedly, we found newborn neurons after SCI with bOEC transplantation, challenging the current central stream theory that there is no neurogenesis after SCI.

Altogether, this thesis provides new insights into the potential of ependymal cells and progenitors during development, regeneration after SCI and after transplantation for SCI treatment, and hopefully can contribute to new therapeutic approaches.

# LIST OF SCIENTIFIC PAPERS

I. **Li, X.***, Floriddia, E. M.*, Toskas, K., Chalfouh, C., Honore, A., Aumont, A., Vallières, N., Lacroix, S., Fernandes, K.J.L., Guérout, N., & Barnabé-Heider, F. (2018). FoxJ1 regulates spinal cord development and is required for the maintenance of spinal cord stem cell potential. *Experimental cell research, 368(1), 84-100*.

II. **Li, X.***, Floriddia, E. M.*, Toskas, K., Fernandes, K. J., Guérout, N., & Barnabé-Heider, F. (2016). Regenerative potential of ependymal cells for spinal cord injuries over time. *EBioMedicine, 13, 55-65*.

III. **Li, X.***†, Honore, A.*, Habib N.*, Chalfouh, C., Delarue, Q., Giovanni M.D., Vaudry, D., Marie, J.P., Barnabé-Heider F., Regev, A.†, Guérout, N.† (2018). The transplantation of olfactory ensheathing cells promotes endogenous stem and progenitor cell reactivity and induces neurogenesis after spinal cord injury. *Manuscript*

(* Co-first authors; † Co-correspondence)

Papers not included in this thesis:

IV. Guérout, N., **Li, X.**, & Barnabé-Heider, F. (2014). Cell fate control in the developing central nervous system. *Experimental cell research*, 321(1), 77-83.

V. Wicher, G., Wallenquist, U., Lei, Y., Enoksson, M., **Li, X.**, Fuchs, B., Hamdeh, S.A., Marklund, N., Hillered, L., Nilsson, G., & Forsberg-Nilsson, K. (2017). Interleukin-33 promotes recruitment of microglia/macrophages in response to traumatic brain injury. *Journal of neurotrauma, 34(22), 3173-3182*.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BDNF | Brain-Derived Neurotrophic Factor |
| bHLH | basic Helix–Loop–Helix |
| BMP | Bone Morphogenetic Proteins |
| bOEC | bulbar Olfactory Ensheathing Cell |
| CNS | Central Nervous System |
| CNTF | Ciliary Neurotrophic Factor |
| CSF | Cerebral-Spinal Fluid |
| DFT | Dorsal Funiculus Transection |
| DH | Dorsal Hemisection |
| DMEM/F12 | Dulbecco's Modified Eagle's/Ham's F12 medium |
| E | Embryonic day |
| EGFP | Enhanced Green Fluoresce Protein |
| ESCs | Embryonic Stem Cells |
| FBS | Fetal Bovine Serum |
| FGF | Fibroblast Growth Factors |
| FoxJ1 | Forkhead Box Protein J1 |
| GDNF | Glial cell-Derived Neurotrophic Factor |
| GGF | Glial Growth Factor |
| IGF1 | Insulin-like Growth factor 1 |
| iPSCs | induced-Pluripotent Stem Cells |
| ISH | In Situ Hybridization |
| JAG2 | Ligand Jagged2 |
| MSCs | Mesenchymal Stem Cells |
| NECs | Neural Epithelial Cells |
| NGF | Nerve Growth Factor |
| NSCs | Neural Stem Cells |

| | |
|---|---|
| OPCs | Oligodendrocyte Precursor Cells |
| P | Postnatal day |
| PCR | Polymerase Chain Reaction |
| RA | Retinoic Acid |
| RGCs | Radial Glial Cells |
| SCI | Spinal Cord Injury |
| Shh | Sonic HedgeHog |
| SVZ | SubVentricular Zone |
| VEGF | Vascular Endothelial Growth Factor |
| WT | WildType |

# 1 INTRODUCTION

The central nervous system (CNS) consists of the brain and the spinal cord. The spinal cord serves as a key bridge for the communication between the brain and the body. Notably, it serves both as the final output command for the elaboration of movements, such as walking or breathing, as well as the first input connection for most sensory modalities. When injury occurs to the spinal cord, the lesion partly or completely disrupts the signal transduction along the CNS, leading to permanent functional impairment in motor and sensory system below the lesion, and typically resulting in neuropathic pain, paralysis, and the dysfunction or disorder of the digestion system and reproductive system (Westgren and Levi, 1998). Spinal cord injury (SCI) is currently a chronic incurable disease without clear epidemiology statistics. However, it is estimated that 9.2 to 246 cases per million of the population a year varying from different regions (Li et al., 2016; Siddiqui et al., 2015). The majority of SCI patients are at the age between 10-40 years old at the time of injury (Siddiqui et al., 2015) and the life quality and expectancy of these young people are extensively and deeply influenced (Center, 2015; Li et al., 2016). Recently, new studies based on stem cell therapies have opened up new promising avenues. Indeed, by manipulating endogenous stem and progenitor cells or by cell transplantation after SCI, these cells can contribute to spinal cord regeneration, including tissue repair and functional recovery (Assinck et al., 2017; Gregoire et al., 2015).

Stem cells are cell types with unlimited self-renewal potential and multipotent capacity. Self-renewal is the process that stem cells divide to generate more identical themselves, maintaining the stem cell pool throughout life, while differentiation is the process by which more specialized cells are formed from stem cells. Self-renewal and differentiation occur spontaneously during organ development from embryonic stages to adulthood and can be specifically mobilized under different pathological conditions. In general, the most potent stem cells are found in early embryonic stages, and the stem cell properties decrease during development and aging (Silva-Vargas et al., 2013). Other progenitor cells, on the other hand, do not share all the characteristics of stem cells, but can also contribute to regeneration after SCI (Sabelstrom et al., 2014; Stenudd et al., 2015).

After embryonic development, except for tissues with fast turnover such as skin or intestine (Li and Clevers, 2010), adult stem cells maintain their population by self-renewal with relatively low division rate and rarely generate fast dividing progenitor cells. In the CNS, adult neural stem cells are mainly restricted in the ventricular-subventricular zone of the lateral ventricles, the subgranular zone of the hippocampus in the brain, and in the central canal of the spinal cord, with limited self-renewal and differentiation potential (Seaberg and van der Kooy, 2003). Although stem and progenitor cells give hope for the recovery after SCI or other neurodegenerative diseases, the regeneration capacity of the adult spinal cord is insufficient (Gregoire et al., 2015). Moreover, the capacity of adult stem and progenitor cells after injury is mostly influenced or determined during the developmental process, but the link and the mechanisms are still largely unknown. Therefore, to further

develop new SCI therapies, it is crucial to understand the cellular and molecular properties of stem and progenitor cells during both embryonic development and adult regeneration (Becker and Diez Del Corral, 2015; Gage and Temple, 2013).

## 1.1 THE DEVELOPMENT OF SPINAL CORD

### 1.1.1 Neurogenesis during spinal cord development

The spinal cord comprises the caudal region of the CNS and serves as a bridge, through which the motor and sensory information travel between the brain and the periphery. At the early stage of spinal cord development before embryonic day (E) 9 in mice, neural epithelial cells (NECs) are under fast proliferation and by migrating away, they expand the dorso-ventral and rostro-caudal dimensions of the spinal cord. Later on, NECs start to lose their epithelial properties and acquire features associated with glial cells and termed radial glial cells (RGCs) (Kriegstein and Alvarez-Buylla, 2009). RGCs are found to be neural precursors throughout the whole CNS, and give rise to neurons and glial cells during neural development, following a spatial-temporal manner (generating neurons first, then glial cells later). This cell fate specification process is primarily determined at an early developmental stage, neural patterning, the biological process by which cells in the developing CNS acquire distinct identities according to their specific spatial positions. At the molecular level, the key player of neural patterning is morphogens, soluble secreted signaling molecules from roof-plate or floor-plate that follow gradient distribution. These morphogens include fibroblast growth factors (FGF), retinoic acid (RA), Sonic hedgehog (Shh), bone morphogenetic proteins (BMP) and Wnts, and govern the arrangements of subtypes of neural precursors and direct their cell fate specification (Gurdon and Bourillot, 2001; Kiecker and Lumsden, 2012).

In the dorsal spinal cord, the regulation of neurogenesis is still not well understood, but many experiments have suggested that two of the key players, BMP and Wnts, determine the dorsal patterning. BMP and Wnt proteins are secreted by the roof plate and therefore are expressed in a graded manner from the dorsal (high) to the ventral (low) developing spinal cord. Progenitors respond to a specific concentration of these proteins by the expression or repression of a specific combination of transcription factors (TFs). These TFs, in turn, direct the determination of progenitor cells. Therefore, the dorso-ventral gradient of morphogens directs progenitors to differentiate into six distinct position-dependent domains of postmitotic dorsal neurons (dI1-6) between E10 and 12.5 in mice (Figure 1) and (Alaynick et al., 2011; Liem et al., 1997). Genetic studies showed that disturbed expression of BMP- or Wnt- associated factors, such as Lmx1a, Gdf7, Wnt1/Wnt3a perturb neuronal cell fate determination in different domains in the dorsal spinal cord during development (Chizhikov and Millen, 2004; Lee et al., 2000; Liem et al., 1997; Millonig et al., 2000; Muroyama et al., 2002). However, the study on dorsal spinal cord neurogenesis still faces many challenges. Genetic inactivation of individual TFs often results in normal spinal cord development (Kiecker and Lumsden, 2012) , which is mainly

because the roof plate secretes several types of Wnts and a large number of BMPs, therefore single inactivation can be compensated by the other TFs.

Neurogenesis of ventral spinal cord is relatively better studied than the dorsal half as more TFs have been investigated by the use of transgenic animals. Probably it is because that this system serves primarily as motor control and motor functions and is easier to study for phenotypes. The patterning is essentially regulated by Shh, a morphogen released from the notochord and by the floor plate during ventral spinal cord development. During the patterning of ventral spinal cord, Shh is expressed in a gradient manner, from ventral (high) to dorsal (low) found to be declining when distributed from ventral to dorsal (Chamberlain et al., 2008). As a consequence, segmental expression of the homeodomain and basic helix–loop–helix (bHLH) TFs subsequently engage in cross-repressive interactions to refine domain boundaries. For example, the expression of the homeobox protein IRX3 is repressed by Olig2, and NKX2.2–PAX6 leads to reciprocal repression (Rowitch and Kriegstein, 2010). Moreover, the concentration and exposure time of Shh regulates the expression of distinct TFs during spinal cord development, such as Olig 2 and Nkx2.2, which further direct the specification of subtypes of neurons in the developing ventral spinal cord following a temporal-spatial manner (Dessaud et al., 2008; Dessaud et al., 2007; Lupo et al., 2006). The Shh gradient activates or represses a number of TFs, giving rise to spatially segregated progenitor domains: FP (Foxa2), p3 (Nkx 2.2), pMN (Olig2), p2 (Nkx6.1 and Irx3), p1 (Nkx6.2) and p0 (Dbx1), where the listed genes encode TFs that specify each domain (Alaynick et al., 2011; Guerout et al., 2014). Progenitors in each domain further differentiate into distinct neuronal subtypes, which are traditionally categorized as motor neurons derived from the progenitors in the pMN domain, and V0, V1, V2 and V3 interneurons derived from progenitors in p0–p3 domains respectively (Figure 1). Importantly, each of these genetically or developmentally defined progenitor types give rise to functionally-distinct neuronal types. Indeed, neurons originating from a similar progenitor domain often share similar position, morphology, projection profile, electrophysiological properties. In other words, the progenitor cell origin of a neuron has become a good predictor of its functional properties and functional role in the mature circuit (Alaynick et al., 2011; Kiehn, 2016). More recently, taking advantages of transgenic animal models, studies in developmental biology have shown that each population of interneurons (V0, V1 V2, and V3) can be even further divided into subgroups based on distinct gene expression profiles (Bikoff et al., 2016; Lu et al., 2015). Even though there are overlaps of makers for different subtypes of interneurons, subpopulations of interneurons can now be defined by combinatorial expression of TFs. For instance, V0 interneurons express Pax2, Pax6, and Evx1; V1 interneurons express Pax2, Evx1, Nurr1; and V2 interneurons express Pax2 and Chx10 (Francius et al., 2013).

Nevertheless, the TFs involved in neurogenesis and neurodevelopment is under active studies and characterizations. Forkhead Box protein J1 (FoxJ1), for instance, is a transcription factor mostly involved in ciliogenesis and previously considered as a specific marker for adult ependymal cells in the spinal cord. However, we unexpectedly found that

FoxJ1 is also involved in spinal cord neurogenesis and development. We used FoxJ1 lineage tracing animal models and some of the combinations of TFs mentioned above to identify further the subtypes of neurons derived from FoxJ1 progenitor cells, and we found that FoxJ1 is transiently expressed by the progenitors of V1 and V2 interneurons during embryonic development, which will be further discussed in detail later (Figure1; Paper I/Li et al., 2018).

### 1.1.2 Gliogenesis during spinal cord development

Gliogenesis in the developing CNS takes place after neurogenesis at the later stage of embryonic development after neurogenesis. Recent genetic studies have shown that similar to neurogenesis, most glial cells, including astrocytes, oligodendrocytes, and ependymal cells also differentiate in a spatial-temporal manner (Hochstim et al., 2008; Tsai et al., 2012; Xie et al., 2012).

Astrocytes are the largest population of glial cells in the CNS, which guide the migration of developing axons and play an essential role in releasing signals for synapse formation and function (Barres, 2008; Powell and Geller, 1999; Sofroniew and Vinters, 2010). In the developing spinal cord, the dorso-ventral axis is segmented into distinct domains under the influence of morphogens during neural patterning as described above. After early embryonic neurogenesis, neural tube progenitor domains switch to a glial fate at around E12.5 in mice. In the ventral spinal cord, progenitors from p1, p2, and p3 domains, which give rise to neurons during neurogenesis at E10-E14 in mice, have been shown to be the precursors of three molecularly heterogeneous subpopulations of astrocytes, VA1-3 astrocytes during gliogenesis. These subtypes of astrocytes are specified by the gradients of BMP and Shh, as well as the combinatorial expression of transcription factors Pax6 and Nkx6.1 (Hochstim et al., 2008). After differentiation, VA1-3 astrocytes can be further characterized by their expression of the axon and the neuronal migration factors Slit1 and Reelin, and their migration to the lateral white matter of the spinal cord, mirroring the localization of the progenitors in the p1–3 domains (Hochstim et al., 2008). Furthermore, a segmental model of astrocyte specification and migration was proposed by a recent study. By using a number of transgenic mouse lines, progenitors from different domains expressing distinct transcription factors such as Pax3, Msx3, Dbx1, Olig2, Ngn3, and Nkx 2.2 along the dorso-ventral axis were fate mapped (Tsai et al., 2012). Astrocytes derived from each domain migrate radially, according to the dorso-ventral position of their neuroepithelial precursor. Postnatally, different subtypes of astrocytes have insufficient migratory potential in both intact and injured spinal cord. Therefore, astrocytes are regionally allocated into the spinal cord according to a segmental template with heterogeneous subtypes (Tsai et al., 2012). Besides, our study recently showed that FoxJ1 is transiently expressed by progenitors of specific subsets of astrocytes during embryonic spinal cord development. One subpopulation of FoxJ1 progenitor-derived astrocytes migrates to the lateral white matter, similarly to Reelin-Slit V1-V3 astrocytes. The other

FoxJ1 progenitor-derived astrocytes migrate to the dorsal funiculus, explicitly labeling a subset of astrocytes with unreported origin from Pax3+ astrocytes (Paper I/Li et al., 2018).

Oligodendrocytes are myelin-forming cells in the CNS, differentiated from oligodendrocyte precursor cells (OPCs). Studying the development of oligodendrocytes and OPCs helps to understand the regulations of myelin production and the regeneration capacity in CNS diseases and injuries. Indeed, during the progress of most of SCI, the necrosis and apoptosis of oligodendrocytes, the failure of sufficient production of oligodendrocytes from OPCs, and the continuous myelin degeneration are crucial factors that result in pathology and functional loss. During development, RGCs in the ventricular zone undergo asymmetric division, giving rise to one daughter cell retaining contact with the ventricular and pial surface, and another daughter cell (OPC) migrating to the gray or white matter (Pringle and Richardson, 1993). Even though the origin of OPCs in the spinal cord is not very well known due to the limitation of genetic animal models, OPCs are found to originate from several domains after neural patterning. In the ventral spinal cord, under the effects of BMP and Shh, the transcription factor Olig2 is specifically expressed in the pMN domain and is phosphorylated before and during neurogenesis, which results in the differentiation of progenitors to motor neurons. However, after E12 in mice, Olig2 undergoes dephosphorylation and heterodimerize with neurogenin 2, contributing to the cell fate change from neural to oligodendrocyte fate (Li et al., 2011). Furthermore, the Notch ligand Jagged2 (JAG2), a Shh-regulated factor transiently expressed in motor neuron progenitors (pMNs) leads to motor neuron to OPC cell fate switch (Rabadan et al., 2012). Interestingly, the disruption of Shh signaling does not deplete all OPC production in the dorsal spinal cord, suggesting that FGF or other factors may compensate for the loss of Shh at least partly (Cai et al., 2005; Vallstedt et al., 2005). After the production of OPCs in the ventral domain pMN, the second wave of OPC production starts in the dorsal spinal cord at around E15. This process is determined by transcription factors including Pax7 and Dbx1, potentially from dI3-dI5 domains (Fogarty et al., 2005; Vallstedt et al., 2005). OPCs derived from dorsal and ventral spinal cord are respectively around 20% and 80% during development (Tripathi et al., 2011). From E18 in mice, OPCs start generating mature myelinating oligodendrocytes, reaching a peak at 2-4 weeks after birth and continue until eight months postnatally (Figure 1) and (Rivers et al., 2008).

Ependymal cells are multi-ciliated glial cells lining the ventricular system, forming a continuous cellular barrier between the cerebral-spinal fluid (CSF) and the adjacent parenchyma. The cilia beating of ependymal cells is responsible for CSF flow, brain homeostasis, and normal functions of adult neural stem cells in the spinal cord (Guerout et al., 2014). Ependymal cells have been categorized into three subtypes by their morphological and molecular features (tanycytes, cuboidal and radial ependymal cells) but the developmental process and the functional differences of ependymal cells are largely unknown (Meletis et al., 2008; Robins et al., 2013; Spassky et al., 2005). Factors such as homeobox transcription factor Six3, Numb/Numlike proteins, and SNX27 were suggested to be crucial for ependymal layer formation in the brain (Kuo et al., 2006; Lavado and

Oliver, 2011; Wang et al., 2016b). During spinal cord development, Shh is essential for ependymal cell differentiation, and deletion of Shh leads to the absence of ependymal layer (Yu et al., 2013). Moreover, FoxJ1 is found to be expressed explicitly in adult spinal cord ependymal cells, and have been used as a spinal cord neural stem cell marker (Barnabe-Heider et al., 2010; Meletis et al., 2008). In the developing spinal cord, the first FoxJ1+ ependymal cells appear earliest at E15.5 in mice by expressing FoxJ1 around the ventricular/ central canal, and quickly expand their population and almost entirely occupy the central canal by birth. Around postnatal day 10 (P10), FoxJ1 is restricted to the ependymal cells which have fully formed the ependymal layer around the central canal, displaying the unique stem cell source of the spinal cord (Li et al., 2016) /(Paper II).

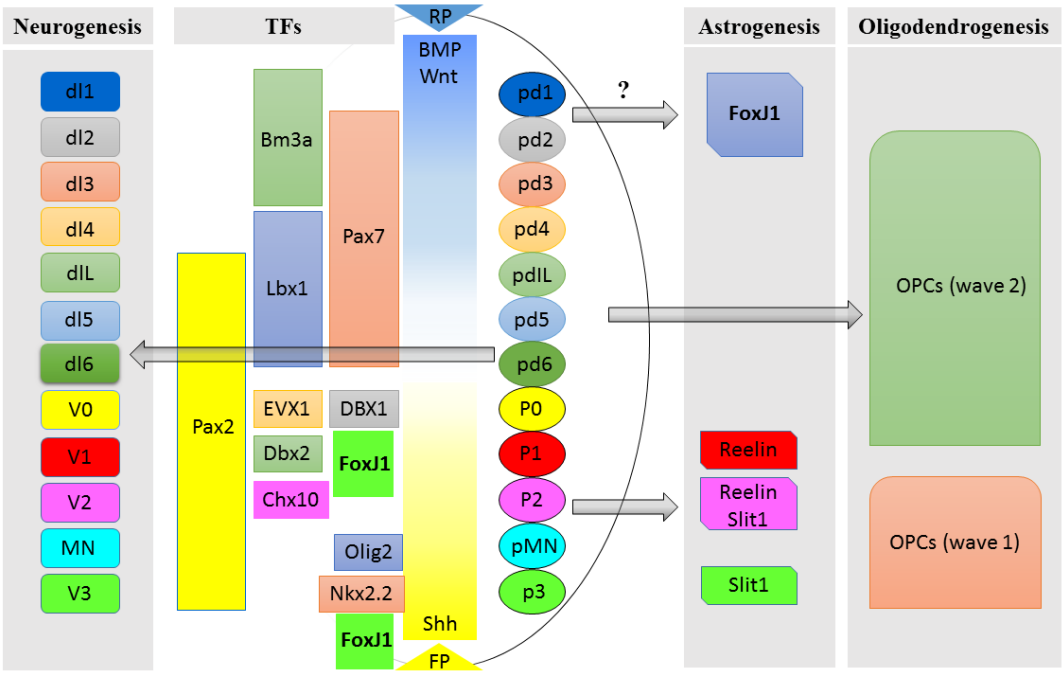



**Figure 1. The developing spinal cord is regulated by morphogens and various transcription factors.** During the development of the spinal cord, morphogens are secreted from the floor plate and roof plate, and distributed in a gradient manner, regulating the expression of a number of transcription factors (middle panel). During neurogenesis, specific transcription factors define domains of progenitors along the dorsal-ventral axis and further give rise to distinct neuronal subtypes (left panel), while other transcription factors in specific domains regulate the development of astrocytes and oligodendrocyte precursor cells during gliogenesis (right panel). The transcription factor FoxJ1 (in bold font with green background), is mainly expressed in the p1-p2 domains and the floor plate during early development and found to be continuously occupying the central canal from E15 onwards, which further regulates the progenitors to give rise to V1 and V2 interneurons, a subset of astrocytes and ependymal cells.

### 1.1.3 The development of spinal cord stem cells

Embryonic stem cells from neuroepithelium of ectodermal origin are the most potent stem cells during early neurodevelopment. However, the stem cell potential starts to decline when neuroepithelial cells begin to specialize into different RGCs around E9-10 in mice. In the brain, adult neural stem/progenitor cells share a common progenitor pool with different types of embryonic progenitors that give rise to cells of different regions of the

brain, including the cortex, striatum, and septum during development. However, this lineage relationship among all the progenitors is lost before E15.5 in mice. Indeed, adult neural stem cells were allocated and specified early in embryonic development, and the lineages between adult and embryonic stem cells diverge during mid-embryonic development (Fuentealba et al., 2015). Moreover, the majority of adult cells with stem/progenitor cell potential in mice are derived between E13.5 and E15.5 in the brain and between E15.5 and P0 in the spinal cord, and remain mostly quiescent in the adulthood (Bond et al., 2015; Fuentealba et al., 2015; Li et al., 2016). Even though these stem cells remain mostly quiescent during postnatal development and adulthood, they can be reactivated under certain conditions, such as injury or stroke (Bond et al., 2015; Fuentealba et al., 2015). In the spinal cord, it is still not clear how the adult neural stem cells obtain their potential during development, but during neurogenesis and gliogenesis, RGCs and other intermediate precursor cells maintain their stem cell potential to both generate their population and give rise to specific differentiated progeny (Temple, 2001). However, the frequency of stem cells is diluted by the production of restricted progenitors and differentiated cells, dropping to 10% at E12 and only 1% at P1 in the spinal cord (Kalyani et al., 1997; Kalyani et al., 1998). The spinal cord stem cell potential is gradually restricted from progenitors to ependymal cells since they begin to appear as differentiated cells at E15. After the first wave of ependymal cells, they quickly take over the full stem cell population within three weeks in mice till around P10 (Li et al., 2016/ Paper II). Using genetic labeling of ependymal cells by FoxJ1-CreER transgenic mice and cell culture studies have shown that the first ependymal cells have stem cell potential as early as E15 while almost 100% stem cells are ependymal cell-derived at P10. This data suggest that embryonic spinal cord stem and progenitor cells from other sources rapidly lose their stem cell potential during development, and the only source of stem cells after birth is confined to ependymal cells around the central canal (Li et al., 2016/ Paper II).

## 1.2 SPINAL CORD STEM/PROGENITOR CELLS AT JUVENILE STAGE AND ADULTHOOD

After embryonic and early postnatal development of the spinal cord, neurogenesis and gliogenesis have been mostly completed. Neurons, astrocytes, oligodendrocytes have been distributed in gray matter or white matter while ependymal cells are restricted around the central canal. Spinal cord astrocytes share similarities with brain astrocytes, including neurotrophic factor secretion, contribution to metabolism and maintenance of homeostasis (Sofroniew and Vinters, 2010). Besides, spinal cord astrocytes are involved in blood spinal cord barrier formation and prevent the influx of antigens or molecule at a certain size to the parenchyma of the spinal cord (Bartanusz et al., 2011). However, unlike subtypes of astrocytes (or GFAP-expressing glial cells) that can give rise to neurons during embryonic development and are involved in adult neurogenesis in the brain, spinal cord astrocytes do not have stem cell potential in both healthy and injured spinal cord (Barnabe-Heider et al., 2010). In the intact adult spinal cord, *in vivo* astrocytes are under low proliferation rate and do not give rise to non-astrocytic progeny. Moreover, fate-mapping experiments showed

that astrocytes form a small number of primary neurospheres but are not capable of being passaged, suggesting that spinal cord astrocytes do not have real stem cell potential (Barnabe-Heider et al., 2010; Buffo et al., 2008). It is still largely unknown how aging affects astrocytes in the spinal cord over time. Previous data about the effects of aging in the brain showed that astrocytes in the neurogenic zone, the subventricular zone (SVZ), are less dividing over time, as well as undergo morphology changes and express a lower level of GFAP, DCX and S100 (Capilla-Gonzalez et al., 2014).

Oligodendrocytes produce myelin, an insulating sheath required for the saltatory conduction of electrical impulses along axons in the CNS. They are derived from oligodendrocyte progenitor cells (OPCs) under physiological condition (Ffrench-Constant and Raff, 1986; Raff et al., 1983a; Raff et al., 1983b). OPCs are distributed in both gray and white matter throughout the CNS. During the differentiation of OPCs into oligodendrocytes, multiple processes are extended, and axons are ensheathed, and then the oligodendrocytes proceed to generate the concentric layers of the modified cell membrane that compose myelin (Sherman and Brophy, 2005). Even though OPCs are proliferating lifelong to generate new oligodendrocytes in mice, it was shown that NG2+ and Olig2+ OPCs are not multipotent and restricted to the oligodendrocyte lineage (Barnabe-Heider et al., 2010; Kang et al., 2010). During aging, the myelination of CNS becomes less efficient, due to both the impairment of OPC recruitment and the differentiation of OPCs to oligodendrocytes, which was found to be associated with the changes of environmental signals and epigenetic changes within OPCs (Hinks and Franklin, 2000; Shen et al., 2008; Sim et al., 2002; Tang et al., 2000).

Ependymal cells are lining the central canal in adulthood with a low proliferation rate *in vivo*. During postnatal development, ependymal cells are contributing to the elongation of the central canal and the flow of cerebrospinal fluid (Alfaro-Cervello et al., 2012; Sabourin et al., 2009). Similarly, ependymal cells at adulthood undergo symmetric division, but all their progeny remain in the ependyma, suggesting that their primary role in adults is ependymal cell maintenance (Barnabe-Heider et al., 2010). Despite the low proliferation rate in the intact spinal cord, it was shown that ependymal cells are capable of generating neurospheres, with the self-renewal potential over many passages (Barnabe-Heider et al., 2010). Moreover, even though ependymal cells do not give rise to other cell types in the intact spinal cord, the neurospheres derived from ependymal cells *in vitro* display multipotent phenotype by differentiating into astrocytes, oligodendrocytes, and neurons (Barnabe-Heider et al., 2010; Li et al., 2018; Li et al., 2016; Meletis et al., 2008). In the aging adult brain, ependymal cells were found to undergo morphology changes, including a higher number of intermediate filaments in the cytoplasm, presenting larger lipid droplets, and concentrated cilia in limited areas (Capilla-Gonzalez et al., 2014). In the spinal cord, the stem cell potential of ependymal cells declines quickly over time from juvenile to adulthood *in vitro*, by displaying less self-renewal capacity and a dramatic decrease of oligodendrocytic differentiation potential (Barnabe-Heider et al., 2010; Li et al., 2018; Li et al., 2016).

The spinal cord is generally considered as a non-neurogenic region in the CNS under normal condition or after injury (Barnabe-Heider et al., 2010; Horner et al., 2000; Shechter et al., 2007). However, a recent study showed that taking advantage of the new technique Div-sequencing which better preserves the integrity of neurons and their RNA content, 19% of the analyzed proliferative cells are immature neurons which can be still detected up to 25 days or longer after survival in the spinal cord. Further analysis showed that these rare newborn neurons in the intact spinal cord are due to GABAergic neurogenesis (Habib et al., 2016). Although the function and the origin of these newborn neurons and the spinal cord neurogenesis capacity between young and aging animals are still not clear, this study opens a new way to study adult neurogenesis and provides more insights on the overlooked neural stem cell potential of the spinal cord.

## 1.3 SPINAL CORD INJURY

### 1.3.1 Scar formation and the response of neural stem/progenitor cells

Spinal cord injury (SCI) leads to massive cell death and a loss of motor function and sensory inputs below the injury level. CNS intrinsic neural cells (neurons, astrocytes, OPCs, ependymal cells), CNS intrinsic non-neural cells (microglia, pericytes, etc.) and immune cells from the blood are affected by and respond to an injury differently (Burda and Sofroniew, 2014). Upon SCI, in the acute phase, the immediate damage of spinal cord leads to the loss of neurons and glial cells, including oligodendrocytes that should be remyelinating the surviving neurons. As a consequence, the loss of neurons after SCI leads to the dysfunction of the motor and sensory system (Ahuja et al., 2017). During the sub-acute phase, inflammation and ongoing necrotic neurons and glial cells lead to the production and secretion of free radicals, chemokines, and cytokines that activate microglial cells (Ahuja et al., 2017). Together with other inflammatory cells such as activated macrophages, polymorphonuclear cells, and lymphocytes, microglia infiltrate the lesion site for further inflammatory response and contribute to ongoing apoptosis of neurons and oligodendrocytes (Ahuja et al., 2017; Hausmann, 2003). After the sub-acute phase of SCI, Wallerian degeneration, an ordered process of axonal death is undertaken (Alizadeh et al., 2015; Waller, 1850). After the injury on the nerve fiber, the axonal skeleton disintegrates, and the axonal membrane breaks apart, which leads to axonal degeneration and the release of myelin debris. Myelin debris has found to be the source of axonal regeneration inhibitors, such as neurite outgrowth inhibitor A (Nogo‑A), oligodendrocyte-myelin, glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) (Filbin, 2003). However, due to the slow infiltration of immune cells and the low capacity of oligodendrocytes to clear myelin, there is accumulation of myelin in the CNS tissue after injury. This accumulated myelin in turn leads to the apoptosis of oligodendrocytes and further contributes to the failure of remyelination and regeneration (Ahuja et al., 2017; Barres et al., 1993; Franklin and Ffrench-Constant, 2008; Vargas and Barres, 2007).

Following SCI, a scar is formed, which is composed of a fibrotic component core and a glial scar surrounding it. The fibrotic lesion core is formed by perivascular cells, including

type-A pericytes (Goritz et al., 2011; Klapka and Muller, 2006; Soderblom et al., 2013), while the glial scar is generated by astrocytes derived from resident astrocytes and ependymal cells-derived astrocytes (Barnabe-Heider et al., 2010; Meletis et al., 2008). The fibrotic scar is traditionally believed to be nonfunctional or have adverse long-term effects and impacts on axon regeneration (Burda and Sofroniew, 2014; Zukor et al., 2013). However, new studies have shown that the absence of type-A pericytes or ependymal cells-derived astrocytes prevents the sealing of wound and therefore worsen SCI outcomes (Goritz et al., 2011; Sabelstrom et al., 2013). Moreover, even though it was widely believed that reactive astrocytes migrate to the injury site and contribute to glial scar formation, recent fate-mapping and live imaging studies showed that astrocytes do not migrate to the lesion site after SCI and most of the astrocytes are not migratory after brain injury (Bardehle et al., 2013; Tsai et al., 2012). Instead, astrocytes are under massive proliferation and upregulate the GFAP expression to further participate in the glial scar formation within their previous locations (Barnabe-Heider et al., 2010; Tsai et al., 2012). The function of the glial scar is under debate regarding its role in attenuating axonal regrowth, but it has been shown that the glial scar also serves as a barrier to block inflammation and immune cell infiltration to the lesion and prevent further tissue damage (Faulkner et al., 2004; Herrmann et al., 2008; Okada et al., 2006; Sabelstrom et al., 2013; Wanner et al., 2013). By impairing the formation of glial scar by transgenic mouse models to block cell cycle of astrocytes-producing ependymal cells or to kill proliferative astrocytes, previous studies have shown that the significant loss of glial scar leads to worsened secondary injury to the tissue and the loss of axonal regeneration (Anderson et al., 2016; Sabelstrom et al., 2013).

Neural stem/progenitor cells, including ependymal cells, astrocytes, and OPCs are highly proliferative after SCI and display different cellular responses (Figure 2). Ependymal cells were found to be the only cell type displaying multi-potency after SCI (Barnabe-Heider et al., 2010; Meletis et al., 2008). Ependymal cells rarely divide around the central canal and can only generate a small number of neurospheres in cell culture under physiological condition. After SCI, ependymal cells divide rapidly and differentiate into other glial cells *in vivo*, and these differentiated progenies leave the central canal and migrate to the lesion side. Half of the ependymal cells differentiate into astrocytes in the glial scar and also produce a few oligodendrocytes that myelinate axons (Barnabe-Heider et al., 2010). Moreover, ependymal cells from an injured spinal cord generate a significantly higher number of neurospheres *in vitro* and can be passaged on with higher self-renewal capacity than those from the non-injured condition. Differentiation assays showed that ependymal cell-derived neurospheres have higher potential to generate oligodendrocytes and neurons *in vitro* after SCI. This observation suggests that the stem cell potential of these neural stem cells is activated by SCI, regarding self-renewal and differentiation (Li et al., 2016/ Paper II). Resident astrocytes dramatically increase their proliferation after SCI and upregulate the expression of GFAP near the lesion site and surrounding area, forming part of the glial scar that prevents inflammation and the infiltration of immune cells. However, astrocytes can only give rise to more astrocytes and do not acquire stem cell potential after SCI, neither

*in vivo* nor *in vitro* (Barnabe-Heider et al., 2010). Unlike the astrocyte-derived astrocytes in the glial scar that forms a barrier to inhibit secondary tissue damage, ependymal cell-derived astrocytes contribute to glial scar formation and reside in the center of the scar, surrounding fibroblast-like stromal cells that make up the core of the forming scar (Barnabe-Heider et al., 2010; Camand et al., 2004; Goritz et al., 2011; Krikorian et al., 1981; Sabelstrom et al., 2013; Shearer and Fawcett, 2001; Windle and Chambers, 1950). It was shown that blocking the cell cycle of ependymal cells leads to enlarged cyst at the lesions, resulting in injuries growing deeper and an increased loss of neurons, partly due to the decreased production of neurotrophic factors by ependymal cells. These findings suggest that ependymal cells act as a scaffold to reinforce the injured spinal cord by restricting secondary enlargement of lesions (Sabelstrom et al., 2013). OPCs are the most proliferative glial cells in the intact spinal cord, and their proliferation is even more increased after SCI. OPCs and ependymal cells generate remyelinating oligodendrocytes after SCI, but OPCs cannot self-renew *in vitro* and do not give rise to other cell types *in vivo*. However, recent studies using transgenic lineage tracing mouse models showed that OPCs are the major cell type that contributes to new myelin formation following SCI (Hesp et al., 2015). OPCs also produce the majority of myelinating Schwann cells in the injured spinal cord, while the contribution to myelination by invading peripheral myelinating Schwann cells after SCI is very limited (Assinck et al., 2017).

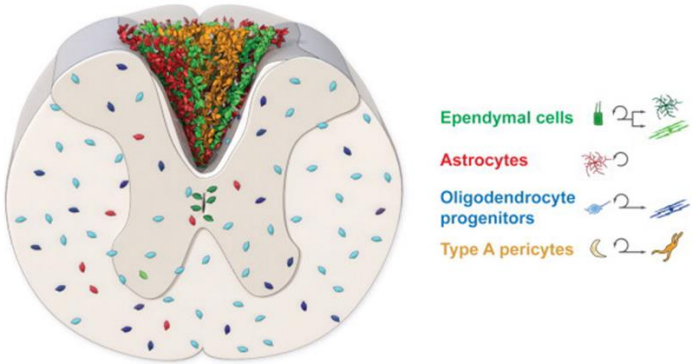



**Figure 2. The response of endogenous cells after SCI (dorsal funiculus lesion).** After a dorsal funiculus lesion, the glial scar is formed with cells produced by ependymal cells (green), astrocytes (red) and pericytes (yellow). After SCI, the ependymal cells self-renew, differentiate, and the progeny migrate to the lesion site. Astrocytes (red), OPCs (blue) and pericytes (yellow) also self-renew and give rise to astrocytes, oligodendrocytes and stromal cells, respectively. Adapted from Sabelstrom et al., 2014.

### 1.3.2 The regenerative potential of spinal cord cells after SCI over time

Even though neural cells intensively respond to SCI with their specific potential, the self-recovery potential of the spinal cord after injury seems to decline during aging (DeVivo et al., 1990; Furlan et al., 2010; Wyndaele and Wyndaele, 2006). It was reported that the potential of neural stem cells decreases during aging and in aged animals after traumatic brain injury, which is due to the quiescence but not loss of neural stem cells, and also due to the change of their differentiation potential (Bouab et al., 2011; Conover and Shook, 2011; Sun et al., 2005). Indeed, astrocytes and ependymal cells significantly change their

morphology and molecular signatures during brain aging, and their proliferation capacity decreases over time (Capilla-Gonzalez et al., 2014). After SCI, there is a higher expression of astroglial and inflammatory markers near and at the lesion site in the aged animals, and the mammalian CNS undergoes an age-dependent decline in axonal regeneration and becomes less regenerative (Geoffroy et al., 2016).

Besides the changes in the microenvironment, the intrinsic regenerative potential of stem/progenitor cells is also age-dependent. At a very young age (P10), all the spinal cord stem cell potential is entirely confined to ependymal cells, but the self-renewal capacity significantly decreases at the juvenile stage and even more in adulthood in mice. The differentiation capacity of ependymal-derived neurospheres to oligodendrocytes also declines over time (Li et al., 2016). Similarly, the recruitment and differentiation capability of OPCs in aged animals largely decrease (Hinks and Franklin, 2000; Kuhlmann et al., 2008; Sim et al., 2002). These findings suggest that the self-recovery capacity decreases with increased aging is partly due to the decreased endogenous remyelination potential by ependymal cells and APCs (Figure 3; Paper II).

After the insult to a spinal cord, astrocytes, ependymal cells and type A pericytes rapidly proliferate and contribute to the scar formation in adult (Barnabe-Heider et al., 2010; Goritz et al., 2011; Sabelstrom et al., 2013). At a younger age, these cell types are more proliferative and pro-regenerative compared to adult cells. After SCI, the stem cell potential of juvenile ependymal cells is more activated than those in adults *in vitro*, by showing greater self-renewal capacity and more oligodendrocyte differentiation. We found that similar lesions are sealed more efficiently in young animals compared to adults. Juvenile lesions have a smaller fibrotic core and smaller glial scar, as well as less infiltration of microglia and blood-derived macrophages (Figure 3). Interestingly, even though ependymal cells have a higher stem cell potential in juvenile mice, they are acting as a backup reserve and contribute to scar formation only when the lesion is larger and need more cells to be sealed (Li et al., 2016/ Paper II). Ependymal cells are required for restricting enlargement of the lesion in adult (Sabelstrom et al., 2013), but the same transgenic mouse model in which the cell cycle of ependymal cells is blocked showed a different phenotype in juvenile animals. The juvenile spinal cord is sealed so efficiently by other cells that blocking ependymal cell proliferation does not lead to deeper lesions nor the formation of a cyst at the lesion site. The area of glial scar and the lesion core are also smaller even when ependymal cells are not able to proliferate. This analysis is in line with the clinical studies that have shown that juveniles have better functional recovery than adults in human (DeVivo et al., 1990; Furlan et al., 2010; Wyndaele and Wyndaele, 2006).

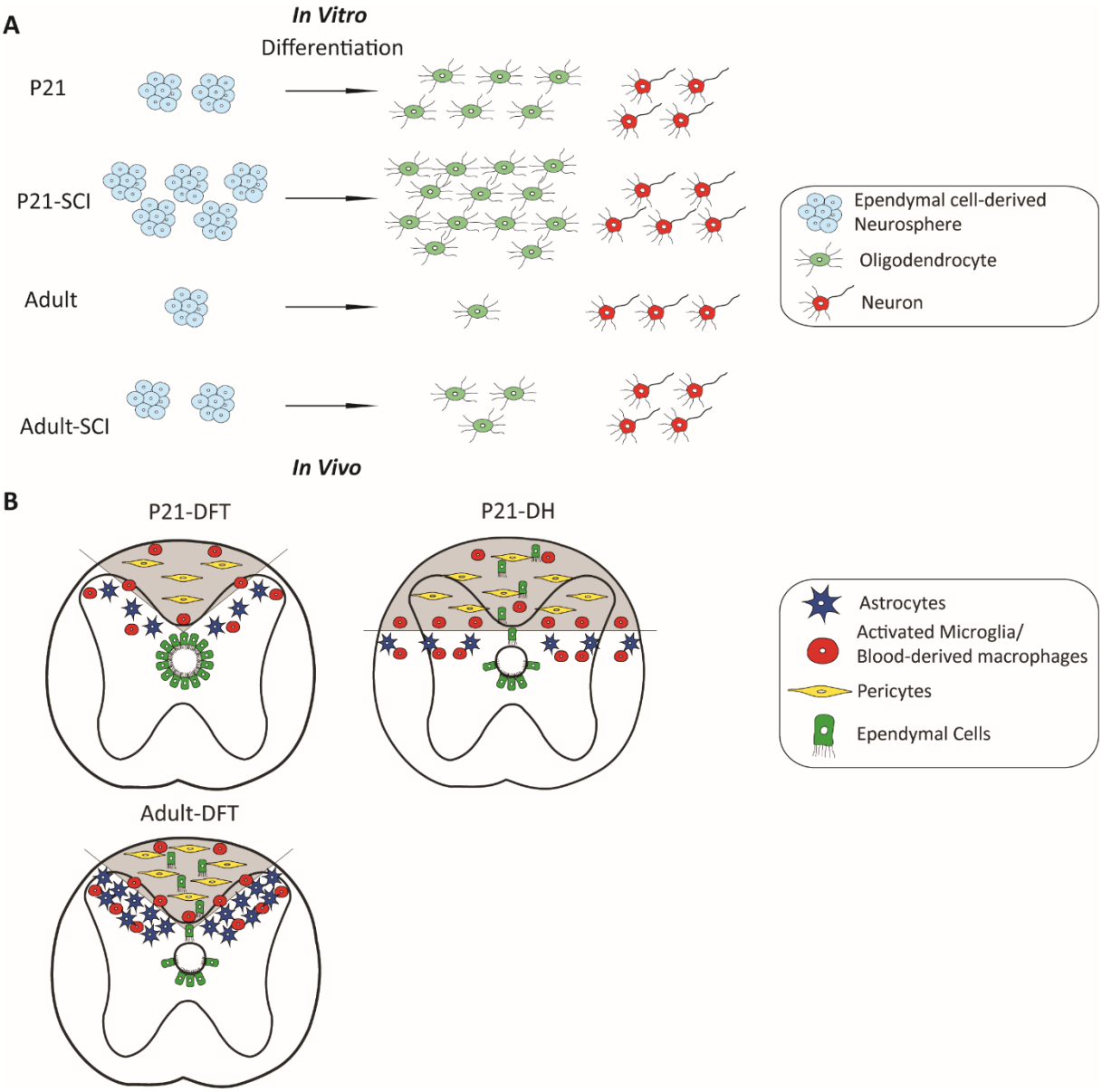



**Figure 3. Ependymal cells and resident cells respond to SCI.** A) Under both physiological and injured conditions, ependymal cells show different self-renewal and differentiation potential in juvenile mice compared to adult mice *in vitro*. B) The response of ependymal cells (green) is age- and lesion size-dependent. Response to SCI of other endogenous cell types is illustrated as follows: resident astrocytes (purple), immune cells (red) and pericytes (yellow). Adapted from Li et al., 2016.

## 1.4 REGENERATIVE APPROACHES

### 1.4.1 Modulating endogenous stem/progenitor cells

Endogenous glial cells play important roles in maintaining homeostasis of the spinal cord. Even though OPCs are the most proliferative cell type throughout the entire parenchyma of the spinal cord, astrocytes and ependymal cells are also under massive proliferation after SCI and contribute to the self-repair of the spinal cord.

As astrocytes are the most numerous cells in the spinal cord, their function as glial scar forming component and their effects on recovery have been under substantial investigation. However, the potential and benefits of clinical applications by modulating astrocytes after SCI are still under debate. Indeed, the glial scar has been long time considered as a barrier for axonal regeneration by inhibiting axonal regrowth (Fawcett, 2006; Okada et al., 2006; Sofroniew, 2009), while there is an increasing number of studies showing the beneficial effects of glial scar for tissue repair (Anderson et al., 2016; Burda and Sofroniew, 2014) but the latter studies are still facing challenges (Silver, 2016). Using conditional knockout strategies to genetically manipulate genes expressed by astrocytes, such as Stat3 and Soc3, can influence the glial scar formation and functional recovery after SCI with different effects (Herrmann et al., 2008; Okada et al., 2006). Conditional ablation of Stat3 results in limited migration of astrocytes and widespread infiltration of inflammatory cells, neural disruption and demyelination with severe motor deficits, whereas the loss of Soc3 leads to rapid migration of reactive astrocytes to seclude inflammatory cells, enhanced contraction of lesion area and notable improvement in functional recovery (Herrmann et al., 2008; Okada et al., 2006). However, most of the studies above used Nestin or GFAP promoter to genetically modulate almost the entire population of reactive astrocytes after SCI, which makes it difficult to draw specific conclusion. Indeed, recent studies showed that spinal cord astrocytes are very phenotypically and functionally heterogeneous in the CNS and after SCI (Bardehle et al., 2013; Tsai et al., 2012). Blocking the subtype of astrocytes derived from ependymal cells after SCI for instance, leads to a more severe lesion and worsen functional recovery (Sabelstrom et al., 2013). Therefore, further investigation should be undertaken to target different subpopulations of astrocytes and understand the role and composition of the glial scar for a better clinical translation.

As previously discussed in this thesis, after the acute phase of SCI, Wallerian degeneration takes place, which is a process of ordered axonal death, resulting in the continuous death of demyelinating neurons and oligodendrocytes (Ahuja et al., 2017; Waller, 1850). Therefore, Wallerian degeneration leads to functional loss after SCI and remyelination have been proposed as a crucial strategy for functional recovery (Plemel et al., 2014). Fate mapping experiments have shown that OPCs are under massive proliferation and differentiate into mature oligodendrocytes after SCI, and contribute to remyelination (Assinck et al., 2017; Barnabe-Heider et al., 2010; Hesp et al., 2015; Sellers et al., 2009). Moreover, NG2+ OPCs undergo substantial proliferation after SCI and generate myelin for several months in rats (Hesp et al., 2015), consistent with another study showing that OPCs from PDGFRα-driven reporter mice start to produce myelin from one month and up to three months after SCI (Assinck et al., 2017). These studies suggest that modulating the proliferation, differentiation and remyelination efficiency of OPCs could be a way to improve functional recovery. However, the spontaneous reactivity of OPCs is apparently not sufficient to obtain functional recovery. Moreover, age affects the recruitment and differentiation of OPCs. A recent study showed that rejuvenation of OPCs restored the regeneration capacity in mice due to the induced youth environment, which suggests that

remyelination-enhancing therapies targeting endogenous cells can be effective throughout life (Ruckh et al., 2012). Even though the mechanisms behind oligodendrogenesis and remyelination are still under investigation, many studies showed that growth factors playing a role in promoting proliferation of OPCs during development could be useful after SCI, such as basic fibroblast growth factor (bFGF), insulin growth factor 1 (IGF1), ciliary neurotrophic factor (CNTF), glial growth factor (GGF) (Plemel et al., 2014). Furthermore, treating mice after with GGF or GGF/FGF increases not only OPC proliferation but also results in improved functional recovery in injured mice (Whittaker et al., 2012). Other strategies, such as targeting extracellular inhibitors of remyelination, inflammatory cells and hormones are also suggested by many studies for potential therapeutic applications (Plemel et al., 2014).

Ependymal cells have been discovered as the only endogenous stem cells in the spinal cord from P10 to adulthood and were suggested as a cell type that can be modulated for non-invasive therapies without the risk of surgical or immune-rejection complications (Barnabe-Heider et al., 2010). Furthermore, as the stem cell potential of ependymal cells has been fully developed since early postnatal age, ependymal cell-based therapies may be used regardless of the age of patients (Li et al., 2016/ Paper II). As ependymal cells are capable to differentiate into astrocytes that are required for reinforcing the injured spinal cord (Barnabe-Heider et al., 2010; Sabelstrom et al., 2013) and give rise to oligodendrocytes that may benefit remyelination (Barnabe-Heider et al., 2010), it gives hope that modulating ependymal cells may contribute to the development new therapies. Even though the molecular mechanism behind the response of ependymal cells to SCI is still mostly unknown, recent studies using transgenic mice have shown that proteins such as FoxJ1 and β1-Integrin expressed by ependymal cells regulate the stem cell potential or the contribution of ependymal cells to glial scar formation (Li et al., 2018; North et al., 2015). Therefore, it is worthy to investigate this cell type further and consider targeting them for gene and cell therapies.

### 1.4.2 Cell transplantation therapies

Even though studies on endogenous stem cells and progenitor cells have shown beneficial effects in experimental and pre-clinical studies, the mechanisms behind these findings need further elucidation. Cell transplantation, on the other hand, has been extensively studied since 20 years (Houle and Reier, 1988; Kunkel-Bagden and Bregman, 1990; Liu et al., 2000; Ramon-Cueto, 2000). Although there are a number of challenges for clinical applications, cell transplantation-based therapies have given some promising results for patients with SCI.

One of the most common cell types for transplantation in SCI studies is mesenchymal stem cells (MSCs). MSCs can be isolated from different sources, including bone marrow, umbilical cord, amniotic liquid and adipose tissue. Advantages of using MSCs include the possibility of autologous transplants to avoid rejection and immune-suppression. Moreover, this approach overcomes ethical concerns compared to the use of embryonic

stem cells (Dasari et al., 2014). Animal studies have shown that MSC transplantation plays an anti-inflammatory protective role and suppresses the activity of immune cells (Caron et al., 2016; Chua et al., 2010; Neirinckx et al., 2014). Further cellular damages by SCI can be reduced as a result of MSC transplantation, partly due to the secretion of trophic factors by MSCs, including vascular endothelial growth factor (VEGF), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (Neirinckx et al., 2014). Clinical trials have also shown that SCI patients after MSC transplantations gain motor and sensory improvements based on the clinical score of American Spinal Injury Association, electromyography and magnetic resonance imaging (Dai et al., 2013; El-Kheir et al., 2014; Jarocha et al., 2015; Jiang et al., 2013; Li et al., 2015).

Embryonic stem cells (ESCs) could compensate for the massive cell loss after SCI by their multipotency to differentiate into neurons and glial cells. ESCs for transplantation therapies can be obtained from embryonic tissues or clonally derived from ESC cultures. ESCs are capable of differentiating into neuronal cells and glial cells upon transplantation, as well as produce factors that can prevent further damage and sustain endogenous tissue regeneration. Animal studies showed that transplanted ESCs can differentiate into neurons (Iwai et al., 2015; Johnson et al., 2010; Salewski et al., 2015; Yang et al., 2013) or oligodendrocytes (All et al., 2015; Sun et al., 2013) in SCI models, and promote significant motor functional recovery (Iwai et al., 2015; Johnson et al., 2010; Salewski et al., 2015; Yang et al., 2013). A clinical trial using oligodendrocytes-derived from ESCs for SCI patients was planned, but the study was terminated due to fund issues (Frantz, 2012). Recently, a new protocol showed that using human ESC-derived neural stem cells can differentiate into diverse neuronal and glial fates *in vitro*, and that functional recovery after SCI is improved significantly after grafting the derived NSCs to the rats with SCI (Kumamaru et al., 2018). Despite the significant efficacy of ESC transplantation in pre-clinical studies, ethical concerns and the uncontrollable side effect of teratoma formation limits the potential clinical applications of ESCs (Herberts et al., 2011; King and Perrin, 2014).

Neural stem cells (NSCs) can be isolated from stem cell niches in the CNS, including subventricular zone and the hippocampus of the brain, and the central canal of the spinal cord (Weiss et al., 1996). Transplanting NSCs into injured spinal cords improved highlimb motor and sensory recovery, and neuronal cell replacement was able to reduce the loss of neural cells with the support of trophic factors (Hawryluk et al., 2012; Hofstetter et al., 2005). Moreover, the first preclinical trial was recently performed with NSC cell line NSI-566, which is derived from a single post-mortem spinal cord of an 8-week gestational age fetus (Curtis et al., 2018). It has been shown that the transplantation of NSI-566 cells into both SCI patients and animals is safe and no serious adverse events were found. Two patients with transplantation treatments had a certain level of functional recovery (Curtis et al., 2018). Even though clinical trials have shown that NSC transplantation into the injury site of SCI patients is safe, it has not been well documented for recovery outcomes in a

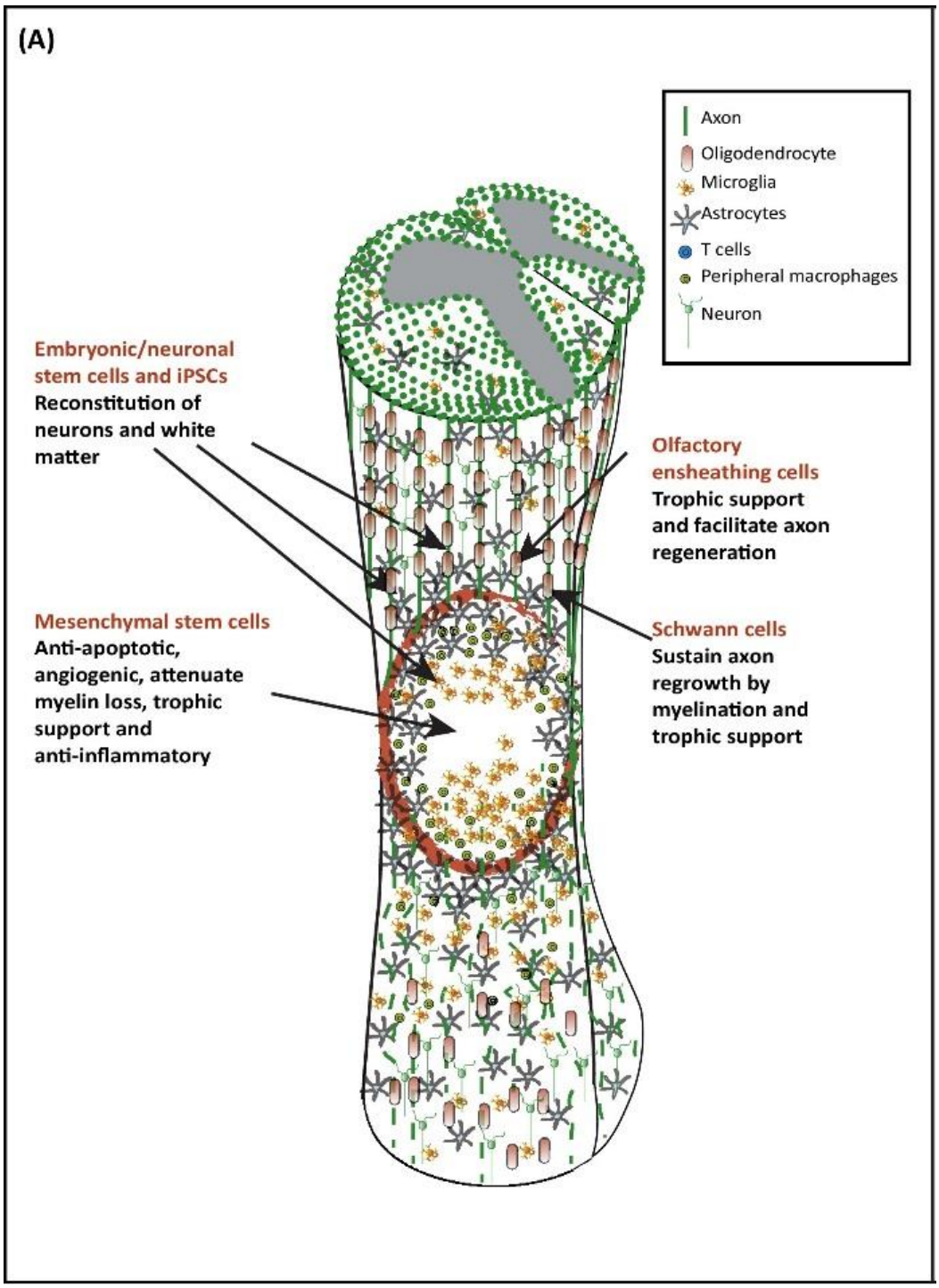
large scale, and there was a lack of control group; thus further studies need to be done (Curtis et al., 2018). Moreover, these NSCs cannot be obtained from the same patient, leading to heterologous transplantations and the need for immunosuppression (Barnabe-Heider and Frisen, 2008; Curtis et al., 2018).

The discovery of induced-pluripotent stem cells (iPSCs) has provided promising medical applications with a lower risk of rejection and without leading to serious ethical concerns regarding the use of embryos. Recent animal studies have shown compelling evidence that iPSC transplantation or iPSC-derived NSC transplantations are safe. Grafted cells give rise to neuronal cells and oligodendrocytes and integrate into the neural network, and some of the studies show motor recovery after transplantation (All et al., 2015; Kawabata et al., 2016; Kobayashi et al., 2012; Lu et al., 2014; Nori et al., 2011; Romanyuk et al., 2015; Salewski et al., 2015). However, other studies suggested that further investigations need to be carried out before clinical trials, as iPSCs and ESCs share similar characteristics and may lead to teretoma formation (Barnabe-Heider and Frisen, 2008; Khazaei et al., 2016).

Besides transplantation strategies using stem cells for beneficial molecules or the replacement of lost cells, autologous glial cell transplantation also leads to improved outcomes after SCI. Specifically, olfactory ensheathing cells (OECs) which are located in the olfactory system are one of the candidates. OECs support and guide axonal growth from the peripheral nervous system to the CNS during development, adult neurogenesis or axonal regeneration. Therefore, it has been hypothesized that OECs could facilitate CNS axon regrowth after injury (Barnett and Chang, 2004; Barnett and Riddell, 2007; Franssen et al., 2007). OECs can be obtained from the olfactory bulb or olfactory mucosa of the same patient of SCI. Animal studies have shown that transplantation of OECs after SCI in rodents promotes functional recovery (Lakatos et al., 2000; Polentes et al., 2004; Ramon-Cueto et al., 1998; Stamegna et al., 2011; Yamamoto et al., 2009). The mechanisms behind these observations are probably because OECs secrete many neurotrophic factors, such as BDNF, GDNF, VEGF, NT-3, etc (Yang et al., 2015). Even though OECs from different sources can promote axonal regeneration and neuroprotective effects at different extent after being transplanted into the injured spinal cord, subpopulations of OECs display different cellular and molecular characteristics (Guerout et al., 2010; Honore et al., 2012). The observations may further lead to variations in the transplantation effects. Indeed, transplantation of OECs obtained from different regions leads to various recovery outcomes: for instance, olfactory bulb-derived OECs have been shown to have better effetcs on motor nerve repair (Paviot et al., 2011). Recent clinical trials showed that transplantation of OECs to a patient, who suffered traumatic transection of the thoracic spinal cord at upper vertebral level Th9, induced axon elongation and functional recovery (Tabakow et al., 2013; Tabakow et al., 2014). Another clinical study, using mucosa-derived OEC transplantation, reported enhanced recovery but with profoundly different scores based on individuals (Wang et al., 2016a). As OECs are highly differentiated cells in comparison to ESCs, OEC transplantation upon SCI overcomes the risk of teretoma

formation (Gomez et al., 2018). The main strategies using cell transplantation therapies upon SCI is summarized in Figure 4 below.

Even though many studies have given promising insights into the mechanism of effects by which OEC transplantation affects SCI recovery positively regarding factor secretion and axonal guidance, little is known about the effects on the regenerative capacity of endogenous stem/progenitor cells after OEC transplantation. Using the transgenic mouse line FoxJ1-CreER$^{T2}$-YFP to specifically fate map and trace ependymal cells and their progeny, we found that transplantation of olfactory bulb-OECs (bOECs) after SCI can enhance proliferation of ependymal cells *in vivo* and self-renewal capacity *in vitro*. Furthermore, bOECs promote astrocytic differentiation from ependymal cells but reduce the expression of axonal regrowth inhibitors, such as CSPGs and Neurocan. Using FoxJ1-CreER$^{T2}$-Rasless mice to block the cell cycle and the proliferation of ependymal cells specifically, we found that bOEC can rescue the enlarged wound occurring due to the lack of the contribution of ependymal cell progeny to the lesion site (Sabelstrom et al., 2013; Paper III). Interestingly, we found that bOEC transplantation can promote adult neurogenesis after SCI, which has not been reported before (Paper III). Our data suggest that bOEC transplantation stimulates endogenous stem/progenitor cells, leading to beneficial effects on recovery of the injured spinal cord. Further studies are carried out to understand the molecular mechanisms occurring after bOEC transplantations, which could be modulated to increase their recovery efficiency.


(A)
Axon
Oligodendrocyte
Microglia
Astrocytes
T cells
Peripheral macrophages
Neuron
Embryonic/neuronal stem cells and iPSCs
Reconstitution of neurons and white matter
Olfactory ensheathing cells
Trophic support and facilitate axon regeneration
Mesenchymal stem cells
Anti-apoptotic, angiogenic, attenuate myelin loss, trophic support and anti-inflammatory
Schwann cells
Sustain axon regrowth by myelination and trophic support

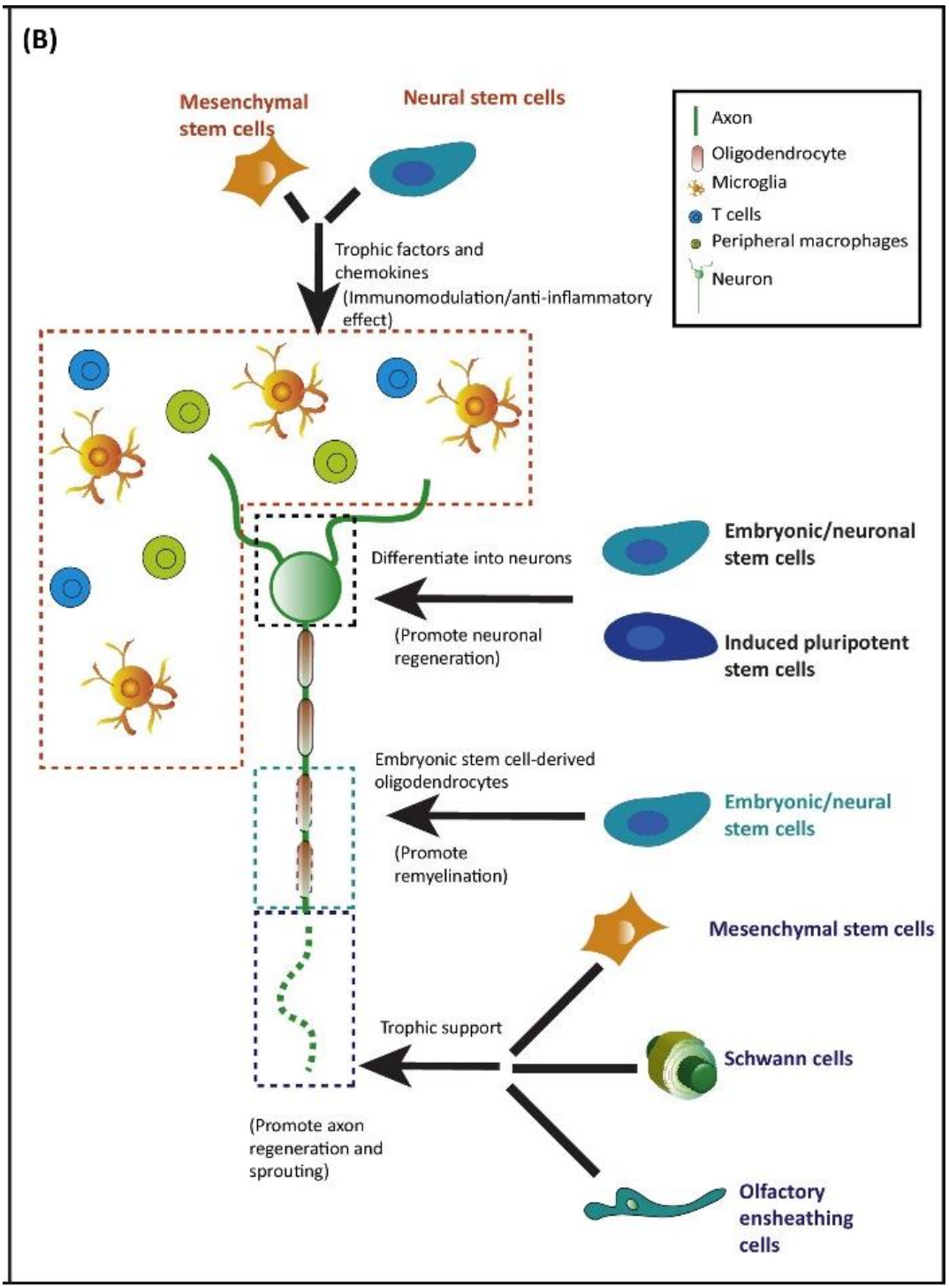



**Figure 4. Strategies using cell therapies for SCI.** A) The main cellular targets and putative mechanism of action of cell-based approaches in mammalian spinal cord after SCI. B) The stem cell-based and glial cell-based mechanisms driving anti-inflammatory and pro-regenerative processes after SCI. Adapted from Vismara et al., 2017.

# 2 AIMS OF THE THESIS

The goal of this thesis is to understand the regenerative potential of endogenous spinal cord stem/progenitor cells during spinal cord development, after spinal cord injury, and after cell therapy. The specific aims are:

- To understand the FoxJ1+ spinal cord progenitors during development and after spinal cord injury;
- To study the different stem cell potential of ependymal cells before and after spinal cord injury from juvenile to adulthood;
- To elucidate the cellular effect of bulbar olfactory ensheathing cell transplantation on the injured spinal cord

# 3 MATERIALS AND METHODS

## 3.1 ANIMALS

All experiments performed in Karolinska Institutet were conducted following the guidelines of the Swedish Board of Agriculture (ethical permit N329/11, N217/14) and were approved by the Karolinska Institutet Animal Care Committee. All experiments conducted in France were under the permit by the French Ministry of Agriculture, Agrifood, and Forestry and were approved by the committee of the French Ministry of Higher education and Research.

*FoxJ1::EGFP*

We used FoxJ1::EGFP mice to label FoxJ1+ cells in the developing spinal cord. In this transgenic mouse line, enhanced green fluoresce protein (EGFP) is driven by the human FoxJ1 promoter without tamoxifen induction (Ostrowski et al., 2003).

*FoxJ1-CreER$^{T2}$-YFP*

We used tamoxifen-inducible FoxJ1-CreER$^{T2}$-YFP transgenic mice (Barnabe-Heider et al., 2010; Li et al., 2018; Li et al., 2016; Meletis et al., 2008) to lineage trace the FoxJ1+ cells during development, and specifically fate map ependymal cells after SCI. Several neural cell populations highly express the FoxJ1 promoter during spinal cord development and exclusively by ependymal cells in the adult spinal cord.

*FoxJ1-CreER$^{T2}$-KI-TdTomato*

We used a knock-in mouse model to study the effect of FoxJ1 during spinal cord development and after SCI. By inserting a sequence of CreER$^{T2}$, Muthusamy et al. specifically disrupted sequence of an exon of foxj1 gene, which leads to the FoxJ1 knockout effect (Muthusamy et al., 2014). We crossed this mouse model with Rosa26-TdTomato mice to further label the FoxJ1 promoter active cells which are FoxJ1$^{+/-}$ or FoxJ1$^{-/-}$ at specific time points. FoxJ1-/- mice are postnatally lethal due to the loss of FoxJ1 (Muthusamy et al. 2014).

*FoxJ1-CreER$^{T2}$-Rasless-YFP*

We used FoxJ1-CreER$^{T2}$-Rasless-YFP mice by crossing FoxJ1-CreER$^{T2}$, Rosa 26-YFP and Rasless mice where the N-, H-ras genes are homogenously knockout, and K-ras gene is between loxP loci. After tamoxifen administration, the cell cycle of ependymal cells is blocked due to the loss of three ras genes while the ependymal cells are labeled with YFP (Sabelstrom et al., 2013).

*Connexin 30-CreER$^{T2}$-YFP*

We Connexin 30-CreER$^{T2}$-YFP mice to specifically label spinal cord astrocytes after SCI and after transplantation treatments (Slezak et al., 2007). After tamoxifen administration, a

large population of astrocytes in the spinal cord is labeled by YFP, allowing us to lineage trace them and their progeny.

*hGFAP-CreER$^{T2}$-YFP*

We hGFAP-CreER$^{T2}$-YFP mice (Ganat et al., 2006) to label spinal cord astrocytes after SCI and after transplantation treatments, complementary to the Connexin 30-CreERT2-YFP mice. After tamoxifen administration, a large population of astrocytes in the spinal cord is labeled by YFP, allowing us to lineage trace them and their progeny.

*GLAST-CreER$^{T2}$-YFP*

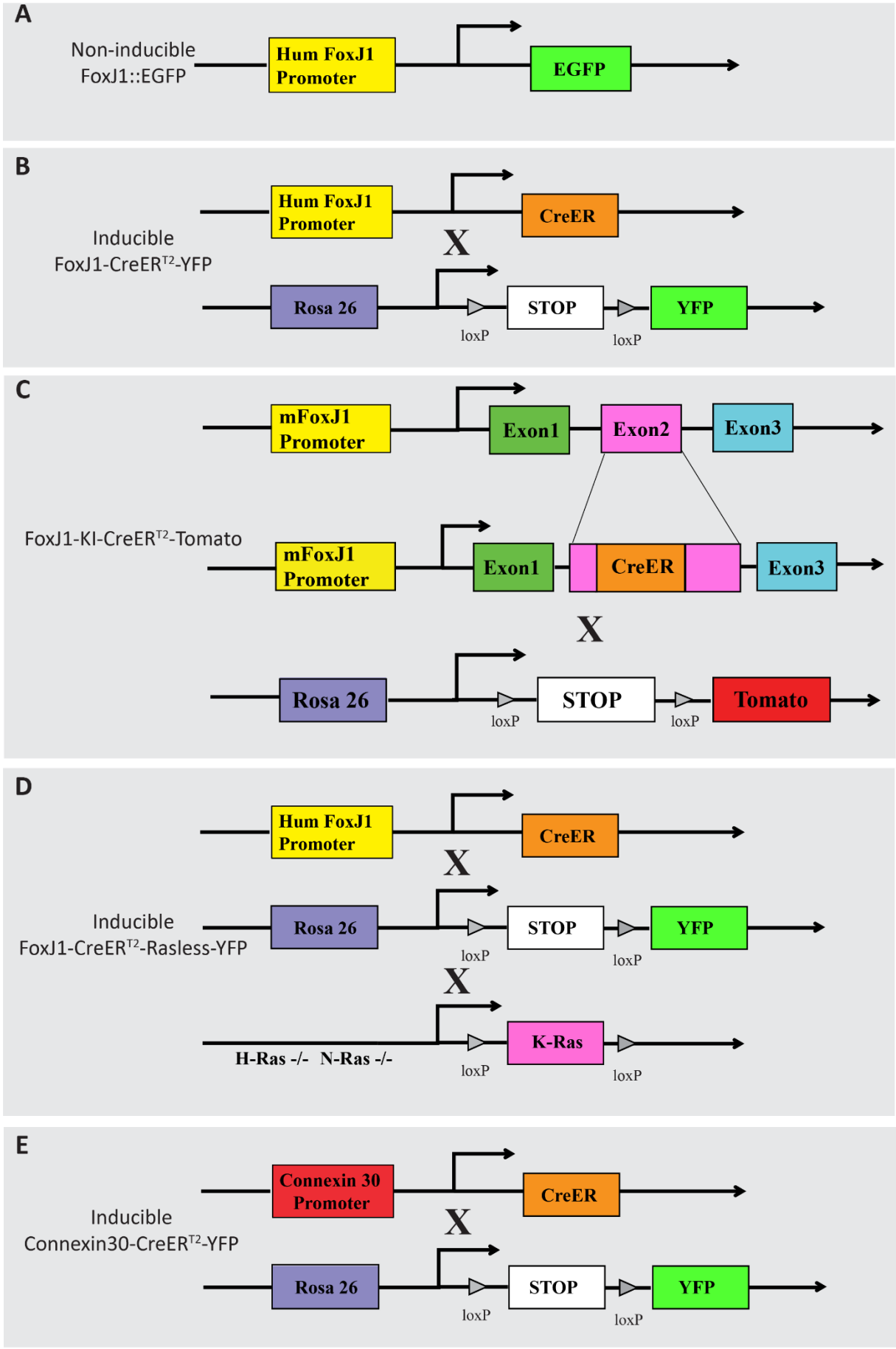
We GLAST-CreER$^{T2}$-YFP mice to label a subtype of astrocytes and progenitors in the spinal cord to study their responses after SCI and after transplantation treatments (Mori et al., 2006). After tamoxifen administration, OPCs in the spinal cord are labeled by YFP, allowing us to lineage trace them and their progeny.

*PDGFRα-CreER$^{T2}$-YFP*

We PDGFRα-CreER$^{T2}$-YFP mice to label spinal cord OPCs after SCI specifically and after transplantation treatments (Rivers et al., 2008). After tamoxifen administration, OPCs in the spinal cord are labeled by YFP, allowing us to lineage trace them and their progeny.

For recombination induction, we injected Tamoxifen at 60 mg/kg of body weight once per day for 5 and 3 days in adult and juvenile mice, respectively. Five days of clearance time allowed the undetectable level of tamoxifen before the start of the SCI experiments. Embryonically and early postnatally, pups' dams were given tamoxifen intraperitoneal injection (60 mg/kg) or gavage to pregnant mice (50 mg/kg) from embryonic day E13-14, E15-17, E17-18, postnatal day P0-P4 and P5-P9. These animals were sacrificed one day after the last injection.

To label proliferative cells and their progeny, EdU (0.075 mg/ml and 1% sucrose) was administered in drinking water, exchanged twice per week and kept in the dark. After spinal cord injury, EdU was given twice by intraperitoneal injections (1.5 mg/ml, 100µl per injection) at 6 hours interval, followed by EdU administration in the drinking water for 7 days.


A
Non-inducible
FoxJ1::EGFP
Hum FoxJ1
Promoter
EGFP
B
Inducible
FoxJ1-CreER$^{T2}$-YFP
Hum FoxJ1
Promoter
CreER
X
Rosa 26
STOP
YFP
loxP
loxP
C
FoxJ1-KI-CreER$^{T2}$-Tomato
mFoxJ1
Promoter
Exon1
Exon2
Exon3
mFoxJ1
Promoter
Exon1
CreER
Exon3
X
Rosa 26
STOP
Tomato
loxP
loxP
D
Inducible
FoxJ1-CreER$^{T2}$-Rasless-YFP
Hum FoxJ1
Promoter
CreER
X
Rosa 26
STOP
YFP
loxP
loxP
X
H-Ras -/- N-Ras -/-
K-Ras
loxP
loxP
E
Inducible
Connexin30-CreER$^{T2}$-YFP
Connexin 30
Promoter
CreER
X
Rosa 26
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YFP
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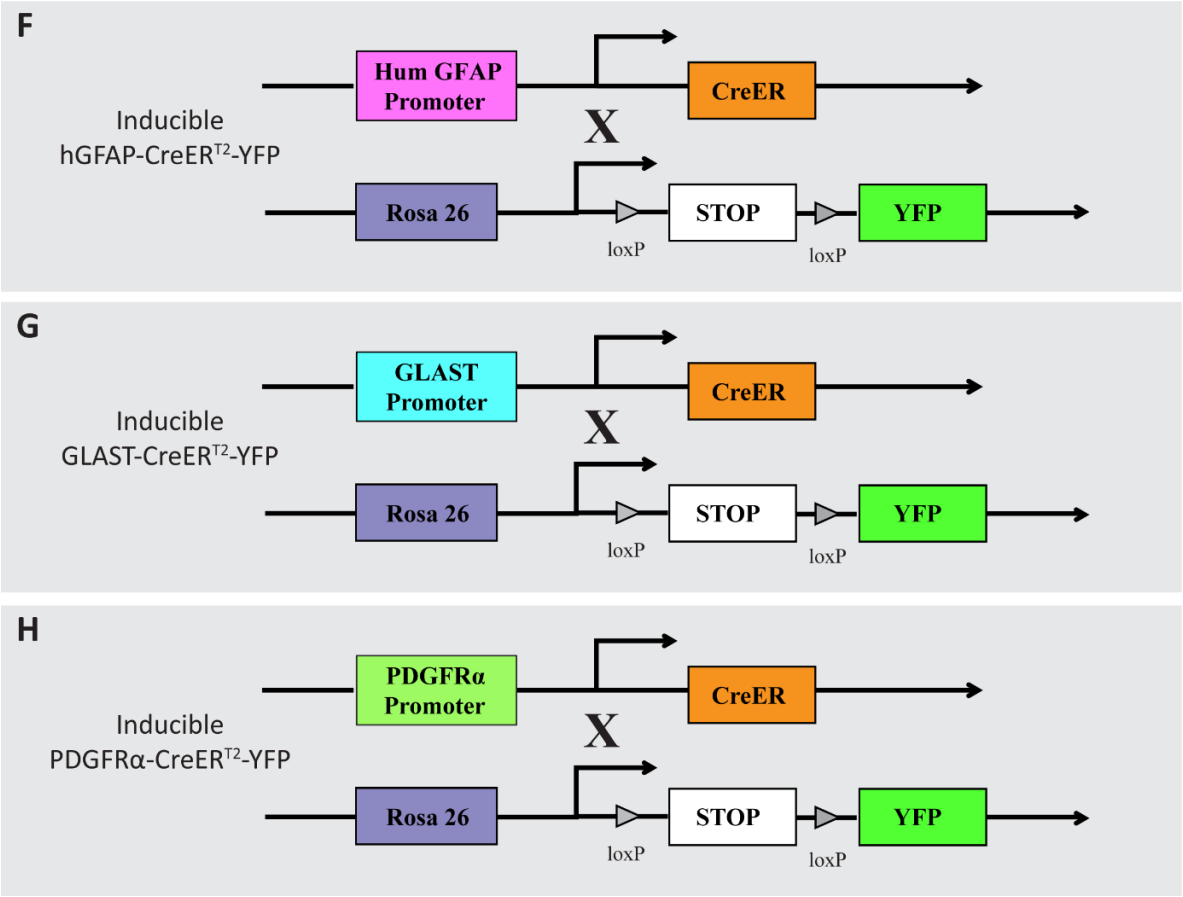


**Figure 5. Schematic illustrations of transgenic mouse models used in this thesis.**

## 3.2 SURGICAL PROCEDURE AND POSTOPERATIVE CARE

### 3.2.1 Spinal cord injury

Mice were kept under anesthesia with a mixture of 5% isoflurane (Baxer) at the starting point then at 2% isoflurane and 1 liter/minute $O_2$ during surgery. Animals were kept on a thermo-pad for the entire surgery to maintain the body temperature around 37-38°C. The back of the animal was shaved and disinfected with 70% EtOH. The skin was incised, the superficial fat gently shifted, and the muscle tissue dissected to expose laminae T9 –T11. Laminectomy was performed at the T10 level, and the dura mater was removed. A dorsal funiculi transection or a dorsal hemisection were performed with a micro knife (FST).

### 3.2.2 Transplantation of olfactory ensheathing cells

Experiments regarding cell transplantation in paper III followed SCI. Bulbar olfactory ensheathing cells were isolated and dissociated manually from 10-day bOEC culture, and approximately 50,000 cells (25,000 cells/ µL with 2 µL/ injection) or DF10S media as control were transplanted into the spinal cord near the midline at around 1 mm depth, 1.5 mm from the midline, 5 mm rostral and caudal to the lesion by a 1 mm sterile glass capillary needle, respectively. Injections were slowly delivered within 1 min, followed by holding the needle at the injection site for 2 min and carefully withdraw the injection needle.

After the surgical process, the wounds were sewed, and mice were placed back in their home cages. Mice underwent a daily check for general health, mobility within the cage, wounds, swelling, infections, or autophagy of the toes. The animals showed neither skin lesions, infection, nor autophagy throughout the study. Bladders were manually expressed after operation until unneeded.

## 3.3 TISSUE PREPARATION AND SECTIONING

For embryo tissues, embryos were collected and fixed in 4% PFA in PBS for at least 12 hours and up to 2 days, then transferred to 30% sucrose solution for at least 2 days up to 1 week. For embryos older than E14, the spines were dissected and isolated after PFA fixation and were transferred to 30% sucrose solution at 4°C for at least 2 days and up to 1 week.

For neonatal mice, pups were decapitated at post-natal day 0 (P0) –P10. Spines were placed in 4% PFA for one day then the spinal cords were dissected out from the spines. The spinal cords were then placed in 4% PFA at 4°C in PBS for 1-2 days, followed by transfer in a 30% sucrose solution and kept at 4°C for at least two days.

For adult spinal cords, the animals were deeply anesthetized with sodium pentobarbital (APL) (150 mg/kg body weight) and perfused transcardially with 0.1 M PBS, pH 7.4, then 4% PFA in PBS, pH 7.4 (Life Technologies). Spinal cords were dissected out from the perfused animals and were further post-fixed in 4% PFA in PBS at 4°C overnight and cryoprotected in 30% sucrose (Life Technologies) for at least 48 h. After embedding in Tissue-Tek OCT compound (Sakura), the spinal cords were cryosectioned sagittally or coronally to 16-20 micron thickness. Sections were collected 1:12 accordingly to stereological principles and stored at -80°C until further use.

## 3.4 IN SITU HYBRIDIZATION (ISH)

ISH was performed to detect mouse FoxJ1 mRNA following previously published method (Pineau and Lacroix, 2007). Basically, a coding sequence of 831 base pairs matching only the Foxj1 gene, as verified by BLAST analysis in Genbank, was amplified by polymerase chain reaction (PCR) from a C57BL/6 mouse brain cDNA library using the same primers used in the Allen Developing Mouse Brain Atlas (forward 5'-ACATCAACTGCCCTGCTACCT-3'; reverse 5'-CTAGCGGGCTTAGAGACCATTT-3'). The amplicon was cloned into the pCR-Blunt II expression vector (Thermo Fisher Scientific Inc.). Radiolabeled cRNA probes were transcribed from linearized cDNAs using the Riboprobe Combination System SP6/T7 (Promega Corporation) in the presence of both [S35]-UTP and [S35]-CTP (Perkin Elmer). Sections of brain and spinal cord were pre-hybridized, hybridized and post-hybridized as previously shown (Pineau and Lacroix, 2007), except for the step in which sections were permeabilized with proteinase K. Instead, a 1.25 mg/mL (~0.0025 U/mL) solution of proteinase K was made and tissue sections digested for 5 minutes for spinal cords and 10 minutes for brains.

All images were acquired using a QIClick™ CCD Camera (QImaging) installed on a Nikon Eclipse 80i microscope.

## 3.5 CELL CULTURE

### 3.5.1 Neural stem cell culture and neurosphere assay

Embryos and postnatal animal were sacrificed for control neurosphere culture. Spinal cord cells were dissociated, and neurosphere cultures were based on the established protocol as described (Li et al., 2018; Li et al., 2016; Meletis et al., 2008).

For the embryonic stages, spinal cords were dissected and dissociated manually, and one small cervical biopsy per animal was kept in 4% PFA for one day then transferred to 30% sucrose solution at 4 °C until further use. Dissociated cells were plated in either in 10 cm petri dish (with 10ml culture medium) or T75 flasks (with 30ml culture medium). Postnatal animals were sacrificed and dissected for the spinal cord. Papain solution and DNase I enzyme were used for cell dissociation (Meletis et al., 2008). All cells isolated from one postnatal animal spinal cord were plated in one 10 cm culture dish (with 10 ml culture medium) or a T75 flask (with 30 ml culture medium), depending on the needs. First neurospheres were collected after two weeks for quantification. Then the harvested primary neurospheres were dissociated manually into single cells for next generations of neurospheres or differentiation. During the passage, approximately 100,000 cells per flask (per animal) were seeded in either 10 cm culture dish or T25 flask. The passaged neurospheres (second, third and fourth generation) were collected and quantified after one week in culture. The differentiation assay is described below. Before the dissociation of the neurospheres, 1 ml medium containing the spheres (4 random selections of 250 ml medium) from each dish/flask were seeded into pre-coated slides (poly-L-lysine or poly-D-lysine) for 12 hours' culture for the analysis of recombination. The groups under the same analysis used the same culture system, including dishes or flasks, coating solution, reagents, etc.

### 3.5.2 Differentiation assay

Dissociated primary neurospheres, approximately 50,000 cells/well or 70,000 cells/well, depending on the proliferation rates of different ages, were plated in poly-D-lysine-coated or poly-L-lysine-coated chamber slides (Sigma) for differentiation with growth factors free medium supplemented by 1% fetal bovine serum. After ten days of differentiation, differentiated cells were fixed with 4% PFA for 20 minutes, followed by immunocytochemistry and data analysis. Two to four independent experiments per group were performed.

### 3.5.3 Bulbar olfactory ensheathing cell culture

The olfactory primary culture was prepared as previously described (Guerout et al., 2010) with slight modifications. Mice were sacrificed by a lethal dose of Thiopental and decapitated. OB was immediately dissected and placed into Hank's buffered salt solution

(HBSS) with 0.1% of trypsin (Invitrogen) after removing meninges. OBs were incubated for 20 min at 37°C. DF-10S medium, including Dulbecco's Modified Eagle's/Ham's F12 medium (DMEM/F12, Invitrogen), supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 0,5% penicillin/streptomycin (Invitrogen) was used to terminate the effects of trypsin. The tissue solution was centrifuged, and pellets were then triturated with a micropipette until a homogenous cell suspension was obtained. Cells were plated in DF-10S in T75 flasks (SARSTED). The flasks were incubated at 37°C, 5% $CO_2$. The medium was changed every two days. Ten days after plating, bOECs were confluent. Before surgery, bOECs were detached from flasks treated with trypsin and the cells were counted with a hemocytometer. The bOECs were resuspended in DF-10S at the concentration of 25 000 cells/µL. Medium culture DF-10S was used for control groups.

## 3.6 IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOCHEMISTRY

*Immunohistochemistry*

Full details of the primary antibodies used are reported in Table 1.

Blocking solution was made with 10% normal donkey serum (Jackson Immunoresearch), 0.3% Triton X 100 (Sigma) in 1X PBS. Blocking solution was applied to tissues for one hour at room temperature. Dilution of primary and secondary antibodies was performed with 2% BSA (Bovine serum albumin; Sigma), 0.2% Triton X100 PBS. Primary antibodies were incubated at room temperature overnight while secondary antibodies were incubated for 1 hour after two times washing with 1X PBS. Secondary antibodies were conjugated with Alexa Fluor fluorophores. Counterstaining was performed with DAPI (1: 10,000) in PBS and sections were coverslipped with Vectashield mounting media (BioNordika). EdU was detected with the Click-iT® EdU Alexa Fluor® 594 imaging kit (Invitrogen) using the Manufacturer’s instructions. Antibodies are described in Table 1. Secondary antibodies (A488, cy3, and cy5) are all from Jackson Immuno Research with host donkey.

*Immunocytochemistry*

After ten days' differentiation assay, differentiated cells were analyzed by immunocytochemistry for quantification. Recombined neurospheres or differentiated cells were stained with primary antibodies at room temperature overnight, followed by secondary antibodies for 1h at room temperature. Antibodies are described in Table 1. Secondary antibodies (A488, cy3, and cy5) are all from Jackson Immuno Research with host donkey.

## 3.7 IMAGE ACQUISITION AND TISSUE ANALYSIS

Confocal representative images of the lesion site and spinal cords were acquired using the Zeiss LSM700 or Zeiss LSM800 microscope set up. Quantification of the lesion and cell infiltration areas was performed using the Zeiss AxioSkop2 microscope set up and AxioVision software.

For embryo samples, regions along the rostral-caudal axis (cervical, thoracic and lumbar area) and different regions in coronal sections, including grey matter, central canal, lateral white matter, dorsal white matter and floor plate were quantified individually. The tissue quantification was performed on at least three sections per domain and per area. For each experimental group and staining, 3-9 animals were analyzed. For SCI samples, the areas were measured at the epicenter of the lesion and sections rostral and caudal to the injury site. SCI and transplantation studies used both coronal and longitudinal sections for analysis. For cell culture analysis, 6-8 randomly selected views per well were used for analysis, and at least three wells per animal were used for statistical analysis. Quantification of the number of cells was performed using the Zeiss Apotome2 microscope set up. The quantification of cells was performed in 2-4 sections per animal. For each experimental group and staining, 3-9 animals were analyzed.

## 3.8 QPCR

Real-time PCR experiments were performed to evaluate the level of mRNA expression of the axon growth inhibitory, axon growth permissive molecules and also neurotrophic factors (Anderson et al., 2016; Sabelstrom et al., 2013). Mice were sacrificed two weeks after SCI, and spinal cord was immediately dissected on ice. Total RNAs were extracted with Tri-reagent (Sigma) and Nucleospin RNAII kit (Macherey-Nagel) according to the manufacturer's protocol. From each sample, 1.5 μg of total RNA was converted into single-stranded cDNA using the ImPromII reverse transcriptase kit (Promega) with random primers (0.5μg/ml). Real-time PCR experiments were performed and monitored by ABI Prism 7500 Sequence Detection System (Life Technologies). The primer pairs used for the different genes analysis have been previously described (Anderson et al., 2016; Sabelstrom et al., 2013).

Mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA was used as the control. Relative expression between a given sample and a reference sample was calculated using the 2-ΔCt method, where ΔCt is the difference in the Ct values for the target gene and the reference gene.

## 3.9 DRONC-SEQ SINGLE NUCLEUS SEQUENCING

Mouse spinal cords two weeks or 3 months after SCI with or without bOEC transplantation were isolated from sacrificed animals and were frozen at -80°C. Samples were shipped on dry ice to Broad Institute, the USA for DroNc-seq, which was described in the published protocol (Habib et al., 2017).

## 3.10 STATISTICAL ANALYSIS

Statistics were run with Student's T-test for comparing two groups and Student's T-test with Bonferroni's correction for more than two groups' comparisons. For all the data analysis, Student's t-test with or without Bonferroni's correction or One-way ANOVA with

Bonferroni's Multiple Comparison was used as indicated. *P<0.05, **P<0.01, ***P<0.001. For each experimental group and staining, 3-9 animals were analyzed.

| Antibody | Species | Dilution | Company (Catalog number) |
|---|---|---|---|
| Chx10 | Sheep | 1:500 | Millipore (AB9016) |
| CNPase | Mouse | 1:200 | Millipore (MAB326R) |
| DAPI | | 1:10000 | Sigma (D9542) |
| Double cortin | Goat | 1:150 | Santa Cruz (sc-8066) |
| EVX1 | Guinea pig | 1:200 | Gift from Dr.Alessandra Pierani |
| Fox3 (NeuN) | Rabbit | 1:500 | Atlas Antibodies (HPA030790) |
| FoxJ1 | Goat | 1:200 | R&D Systems (AF3619) |
| GFAP | Rabbit | 1:500 | Millipore (AB5804) |
| GFP | Chicken | 1:500 | Aves (GFP-1020) |
| Iba1 | Rabbit | 1:500 | Wako (019-19741) |
| Ki-67 | Rabbit | 1:200 | ThermoScientific (RM-9106) |
| Neurocan | Mouse | 1:200 | Sigma (N0913) |
| NG2 | Rabbit | 1:200 | Millipore (AB5320) |
| Pax2 | Rabbit | 1:500 | Life technologies (71-6000) |
| PDGFR-β | Rabbit | 1:200 | abcam (ab32570) |
| Reelin | Mouse | 1:200 | Millipore (MAB5364) |
| Sox10 | Goat | 1:500 | R&D Systems (AF2864) |
| Sox9 | Goat | 1:500 | R&D Systems (AF3075) |

| | | | |
|---|---|---|---|
| Tuj1 | Mouse | 1:500 | Covance (MMS435P) |
| Vimentin | Chicken | 1:500 | Millipore (AB5733) |

**Table1. Primary antibodies used in the studies of this thesis.**

# 4 RESULTS & DISCUSSION

## 4.1 FOXJ1 IS REQUIRED FOR SPINAL CORD DEVELOPMENT AND THE MAINTENANCE OF SPINAL CORD STEM CELL POTENTIAL

FoxJ1 is a transcription factor that is usually considered involved in ciliogenesis during development (Clevidence et al., 1994; Hackett and Weller, 1995; Murphy et al., 1997; Stubbs et al., 2008; Yu et al., 2008). In the spinal cord, FoxJ1 has been widely used as a marker to label ependymal cells, the only spinal cord stem cells in adult mice, to study their potential after SCI (Barnabe-Heider et al., 2010; Li et al., 2016; Meletis et al., 2008; North et al., 2015; Sabelstrom et al., 2013). However, we found that FoxJ1 is transiently expressed in other cell types than ependymal cells in the developing spinal cord. Indeed, using *in situ* hybridization, we found that *foxj1* mRNA expression is not restricted around the central canal, but also along the entire ventricular zone and appears since E10, earlier than the first appearance of ependymal cells at E15. Our FoxJ1 immunostaining on WT mice also shows that FoxJ1 expression is along the ventricular zone, earliest found at E12-E13, suggesting that FoxJ1 is transiently expressed in neural progenitors during development.

To study the migration of FoxJ1+ progenitors during development, we then used FoxJ1::EGFP mice as a short-term fate-mapping tool to label the FoxJ1 lineage cells, as the half like of EGFP is usually longer than transcription factors. We found that the progeny derived from FoxJ1+ progenitors are distributed in gray matter and floor plate since E10, then their distribution expands to central canal, lateral and dorsal white matter during neurogenesis and gliogenesis from E10-E17 with different morphology. However, EGFP+ cells drastically decreased and eventually are restricted to the central canal since P10.

To characterize the cell types derived from FoxJ1 lineage, we used proliferative marker Ki67 and neuron/interneuron markers NeuN, EVX1, PAX2 and Chx10 during neurogenesis, and used other glial markers including Sox9, Vimentin, Reelin, and GFAP during gliogenesis for the EGFP+ cells. The expression pattern of transcription factors of interneuron development shows that Pax2 is widely expressed by various type of V1 interneurons, while EVX1 and Chx10 are neatly expressed in the regions of V0 and V2 interneurons respectively at E10 (Alaynick et al., 2011). We observed that the EGFP+ cells in the gray matter during neurogenesis (E10-13) are V1 and V2 interneurons, as many of them are co-labeled with PAX2 and a small number with Chx10. During gliogenesis (E15-E18), we found that the number of EGFP+ interneurons decreased, but the other EGFP+ cells with increasing numbers are subpopulations of lateral and dorsal astrocytes, as well as ependymal cells. The neural cells of the developing spinal cord are very heterogeneous. Indeed, astrocytes constitute one of the most abundant cell populations in the developing spinal cord and are functionally heterogeneous derived from various progenitors (Tsai et al., 2012). The knowledge of their origins is still very limited. Previous studies showed that progenitors from the ventricular zone differentiate into different subtypes of astrocytes and are in general distributed radially, in accordance to the dorsal-ventral position of their progenitors (Hochstim et al., 2008; Tsai et al., 2012). Our data consistently showed that a

subset of EGFP+ astrocytes migrates to the lateral white matter horizontally with Reelin, Sox9, and GFAP expression, in line with a subpopulation of the astrocytes reported by Hochstim et al. (Hochstim et al., 2008). However, our data complimentarily showed that a subset of EGFP+ astrocytes migrates to the dorsal white matter, as a subpopulation of PAX3+ astrocytes in the dorsal spinal cord (Tsai et al., 2012). We suggest that FoxJ1 is expressed in progenitors of both neuronal and astrocytes, and that FoxJ1 can be further used as a new marker to study neural cells during spinal cord development.

To further understand the role of FoxJ1 in the developing and adult spinal cord, we used FoxJ1-CreER$^{T2}$-YFP and FoxJ1-CreER$^{T2}$-KI-TdTomato mouse lines. We observed that after the loss of one or two alleles of the foxj1 gene, less FoxJ1 promoter-active cells are capable of proliferating with a five-fold decrease, and they leave the progenitor stage earlier by showing less Sox9 and Vimentin expression but become more neurogenic by expressing more interneuron marker Pax2. However, these cells are disrupted for maturation as they are not able to express mature neuron marker NeuN. As previous studies showed that FoxJ1 is involved in the migration and differentiation of progenitor cells towards the olfactory bulbs (Jacquet et al., 2011), our data further expand the understanding of FoxJ1 from brain to the CNS development. Our study suggests that FoxJ1 is involved in the proliferation and is required for normal cell fate specification of progenitors during spinal cord development.

Moreover, we further discovered that FoxJ1 also plays a role in the maintenance of stem cell potential in adulthood besides its function during development. After embryonic development, the stem cell potential is entirely confined to ependymal cells since P10 (Li et al., 2016). Even though we were not able to acquire FoxJ1$^{-/-}$ mice postnatally due to the lethal effects of losing FoxJ1 (Muthusamy et al., 2014), FoxJ1$^{+/-}$ mice showed that the self-renewal potential of ependymal cells was largely impaired at both P10 and adulthood. Indeed, in both FoxJ1-CreER$^{T2}$-YFP and FoxJ1-CreER$^{T2}$-KI-TdTomato mouse lines, the central canals were occupied by FoxJ1+ ependymal cells since P10, but only 60% of the neurospheres derived from the spinal cord were recombined in FoxJ1$^{+/-}$ mice, compared to 90% from the FoxJ1-CreER$^{T2}$-YFP mice (FoxJ1$^{+/+}$), indicating that a large population of ependymal cells does not have stem cell potential after the loss of one copy of FoxJ1. From the second generation of neurosphere culture, we observed that all the neurospheres from both mouse lines were recombined, suggesting that the only stem cell source is still ependymal cells, and the loss of FoxJ1 impairs the self-renewal potential of ependymal cells but does not promote another stem cell niche in the spinal cord. Moreover, differentiation assay showed that the FoxJ1$^{+/-}$ neurospheres are more neurogenic but failed to generate oligodendrocytes, which is in line with our *in vivo* data during development that FoxJ1$^{-/-}$ progenitors are more differentiated and more neurogenic.

Interestingly, our study timely gives more insights into a debate about the contribution of ependymal cells after SCI. Ependymal cells have been shown to be the only cell type with multipotency after SCI (Barnabe-Heider et al., 2010; Meletis et al., 2008), and contribute to the reinforcing of the injured spinal cord by restricting tissue damage and neural loss

(Sabelstrom et al., 2013). However, a recent study contradictorily showed that by using FoxJ1-CreER$^{T2}$-KI-TdTomato mice, the contribution of ependymal cells to scar formation after SCI is very limited (Ren et al., 2017). To test whether the injury could rescue the decreased self-renewal capacity of FoxJ1$^{+/-}$ ependymal cells, we performed the same SCI in both FoxJ1-CreER$^{T2}$-YFP (FoxJ1$^{+/+}$) and FoxJ1-CreER$^{T2}$-KI-TdTomato (FoxJ1$^{+/-}$) mice. We found that the effects of the FoxJ1 deletion on the stem cell potential of ependymal cells are permanent. Indeed, the FoxJ1$^{+/-}$ ependymal cells did not migrate to the injury site as much as the FoxJ1$^{+/+}$ ones, and the FoxJ1$^{+/-}$ ependymal cells showed similar phenotype as those before SCI, regarding decreased self-renewal potential and failure to generate oligodendrocytes. Therefore, our study suggests that FoxJ1 is required for the maintenance of normal stem cell potential of ependymal cells postnatally and after SCI. Further usage of FoxJ1-CreER$^{T2}$-KI-TdTomato mice for lineage tracing might be more limited than what the authors claimed as one copy of FoxJ1 is knocked out (Muthusamy et al., 2014). Therefore, the FoxJ1-CreER$^{T2}$-KI-TdTomato mice need more characterization before used for more analysis.

## 4.2 THE REGENERATIVE POTENTIAL OF EPENDYMAL CELLS FOR SCI OVER TIME

Ependymal cells have been discovered as the only stem cells in the spinal cord and proposed as a target for non-invasive cell therapy for SCI (Barnabe-Heider et al., 2010; Meletis et al., 2008). However, when the ependymal cells have developed stem cell potential and how their potential can change over time were not clear. As the majority of SCI affected people are 10-40 years old at the time of injury (Siddiqui et al., 2015), and the recovery potential of the spinal cord after injury declines during aging (DeVivo et al., 1990; Furlan et al., 2010; Wyndaele and Wyndaele, 2006), it is crucial to understand how the potential of endogenous stem cell changes over time to develop translational therapies for patients of different ages.

Although it was previously suggested that ependymal cells could be targeted for stem cell therapy without invasive surgery, it was not known that when and how the ependymal cells become stem cells. In Paper II, using non-inducible FoxJ1::EGFP mice where EGFP is expressed under the active FoxJ1 promoter, we found that ependymal cells are first born at E15 in mice, and the spinal cord stem cell potential is entirely confined to ependymal cells from early postnatal stage P10, suggesting that treatments modulating ependymal cells for SCI can be applied to patients at almost all ages. Consistent with other studies that the spinal cord become less regenerative with aging (DeVivo et al., 1990; Furlan et al., 2010; Wyndaele and Wyndaele, 2006), we found that the ability of ependymal cells to self-renew declines from juvenile stage to adulthood and this capacity is also less re-activated after SCI. Moreover, ependymal cell-derived neurospheres differentiate into significantly fewer oligodendrocytes in adulthood compared to in juvenile stage, but we found no difference in neuronal differentiation when comparing different ages. It was shown that during aging, neural stem cells lose their stem cell potential after traumatic brain injury (Conover and

Shook, 2011; Sun et al., 2005). Both of our data and previous publications suggest that it would be more beneficial to modulate ependymal cell differentiation after SCI into oligodendrocytic lineage instead of neurons, since ependymal cells do not give rise to neurons *in vivo* at different ages and their neuronal differentiation potential is not age-dependent *in vitro* after SCI (Barnabe-Heider et al., 2010; Li et al., 2016).

Adult ependymal cells are capable of differentiating into astrocytes and oligodendrocytes *in vivo* after SCI, and the differentiated progeny migrate to the lesion area (Barnabe-Heider et al., 2010; Meletis et al., 2008). However, although juvenile ependymal cells have greater self-renewal potential, we found that there are no migration of the ependymal lineage cells to the lesion site after dorsal funiculus transection (DFT), a mild SCI model. On the contrary, after more severe SCI, dorsal hemisection (DH), juvenile ependymal cells and their progeny migrate to the lesion site, suggesting that the juvenile spinal cord environment after injury is different from that of adults. Despite the higher intrinsic stem cell potential, juvenile ependymal cells are not recruited if the injury can be sealed by other cell types.

It has been well studied that after SCI, several endogenous cell types are required to contribute to the wound healing and scar formation in a distinct manner, including ependymal cells, astrocytes and stromal-derived pericytes (Anderson et al., 2016; Goritz et al., 2011; Sabelstrom et al., 2013). Ependymal cell-derived astrocytes are located in the core while resident astrocytes are at the border of the lesion site after SCI (Barnabe-Heider and Frisen, 2008; Burda and Sofroniew, 2014). We observed that both the lesion core (where reactive astrocytes do not infiltrate) and the surrounding area with astrogliosis (where the reactive astrocytes accumulate) in juvenile mice after DFT are smaller than those in adults, indicating that the juvenile spinal cord can seal the lesion site more efficiently than adult after a mild injury. After DH, these areas show no significant difference between juvenile and adults, but juvenile ependymal cells are activated and their progeny is capable to migrate after this more severe injury, further suggesting that the recruitment of ependymal cells to the injury site is dependent on the self-healing efficiency of the spinal cord.

Indeed, our data showed that there is increased activation and infiltration of microglia and blood-derived macrophages in adult mice compared to in juvenile mice after mild injury. The activation and recruitment of these immune cells reached the same level when comparing adult with a mild lesion to the juvenile with a severe lesion. Pericytes are the primary cell type forming the fibrotic cap filling the lesion site after SCI (Dias et al., 2018; Goritz et al., 2011). Consistent with our suggestion that the juvenile spinal cord is more regenerative, we observed that the infiltration by pericytes in injured juvenile mice is significantly less than the one in adults after the same type of SCI. This observation is probably due to the higher repair mechanism of astrocytes at the younger age and the age-dependent transcriptomic changes regarding inflammation or metabolism (Noor et al., 2011; Saunders et al., 2014).

To further understand whether ependymal cells are needed only when the regeneration is largely insufficient, due to aging or a larger injury, we used FoxJ1-CreER$^{T2}$-Rasless-YFP mice to block the cell cycle of ependymal cells. Consistent with a previous study we found that lacking proliferative ependymal cells after SCI in adulthood leads to the formation of an enlarged cyst at the lesion site (Sabelstrom et al., 2013). However, using this transgenic model in juvenile mice, we found no cysts or enlarged lesion sites after DH injury, and the astrogliosis and stromal infiltration areas at the injury site of juvenile mice are significantly smaller than in adults. These data confirm that ependymal cells serve as a backup participant for regeneration after SCI and juvenile spinal cords have a more pronounced potential for self-repair. Notably, in line with our findings, a recent clinical study reveals that younger patients with SCI have better overall health and functional recovery as well as reduced pain (Ma et al., 2016). It is suggested that modulating endogenous cell types would allow the development of non-invasive therapeutic possibilities to avoid the complications associated with cell transplantation (Barnabe-Heider and Frisen, 2008; Coutts and Keirstead, 2008). Therefore, our understanding about age- and lesion size-dependent self-repair of spinal cords provides more clues for future individual therapy development and suggests that adequately tuning the response of ependymal cells and other glial cells should be explored to promote spinal cord repair after SCI.

## 4.3 THE TRANSPLANTATION OF BOECS PROMOTES THE REGENERATIVE POTENTIAL OF ENDOGENOUS PROGENITORS AFTER SCI

Even though the recruitment of endogenous ependymal cells presents a promising potential for the cure of SCI, there need more investigations of its mechanisms before clinical application. On the other hand, cell transplantations are more studied (Assinck et al., 2017; Kabu et al., 2015; Vismara et al., 2017; Yang et al., 2015). Stem cell transplantations, including ESCs, iPSCs, NSCs have shown the potential to compensate for cell loss but are still under challenge due to difficulties to control of their proliferation and cell fates (Assinck et al., 2017). OECs, a glial cell type, however, have shown to be beneficial for SCI recovery in both animal studies and clinical trials with human patients (Mayeur et al., 2013; Tabakow et al., 2014). However, studies focusing on OEC transplantation are usually limited to axonal regrowth and their enrichment of microenvironment (Gomez et al., 2018). Since OEC transplantation achieve such significant functional recovery in clinical trials (Tabakow et al., 2013; Tabakow et al., 2014; Wang et al., 2016a), we hypothesized that the transplanted OECs also have effects on endogenous progenitors and stem cells. In paper III, we used different transgenic mice and single nucleus sequencing techniques to fate map ependymal stem cells and other endogenous progenitors, to further understand the effects of bOEC transplantation on these progenitors.

To investigate the effects of bOEC transplantation on ependymal cells after SCI, we used FoxJ1-CreER$^{T2}$-YFP mice for proliferation, differentiation and neurosphere assays. We found that after SCI, ependymal cells showed higher reactivity in terms of self-renew and neural differentiation compared to non-injured group *in vitro*, similar to what was seen in

previous studies (Barnabe-Heider et al., 2010; Cusimano et al., 2018; Li et al., 2016; Meletis et al., 2008). Indeed, our *in vitro* neurosphere assay also showed that significantly larger number of neurospheres were derived from the same number of ependymal cells after bOEC transplantation, suggesting that the transplantation of bOECs increases the proliferation of ependymal cells and their self-renewal capacity. Similarly, with bOEC transplantation, we found that the proliferation of ependymal cells were even more highly activated by showing a significantly higher percentage of EdU+YFP+ cells *in vivo*.

To study the effects of bOEC transplantation on the progeny of ependymal cells, we analyzed animals two weeks and three months after surgery. We found that bOEC transplantation did not change the oligodendrocytic differentiation potential of ependymal cells, while the transplantation enhanced astrocytic differentiation of ependymal cells, with a higher percentage of GFAP+YFP+ cells. Moreover, the newly derived astrocytes from ependymal cells showed less Neurocan expression *in vivo*, suggesting that bOEC transplantation promotes astrogliogenesis but reduces axonal inhibition. Interestingly, our previous study showed that ependymal cell-derived neurospheres can differentiate into more oligodendrocytes and neurons *in vitro* after SCI (Li et al., 2016), our differentiation assay here showed that the SCI-induced activation of oligodendrocytic differentiation is highly associated with SCI, regardless of bOEC transplantation; whereas the neuronal differentiation potential of neurospheres can be increased with bOEC transplantation, regardless of SCI.

To further investigate why the differentiation potential of ependymal cells are changed upon bOEC transplantation, we further performed qPCR to analyze the micro-environment. Our qPCR data showed that after bOEC transplantation, there was an increase of the mRNA level of a few axonal growth permissive molecules, whereas the mRNA level of mostly axonal growth inhibitory molecules decreased, suggesting that bOEC transplantation enriched the micro-environment by inhibiting the inhibitors of axonal growth. Moreover, neuronal survival was significantly increased with bOEC transplantation, and this effect could be seen from 2 weeks to 3 months after transplantation. Therefore, our data suggested that bOEC transplantation results in the more enriched microenvironment, regarding axonal growth and neuronal survival. As ependymal cells are also crucial to reinforce the spinal cord after SCI by serving as a source of neurotrophic support for neuronal survival and wound healing (Sabelstrom et al., 2013), we further investigated the effects of bOEC transplantation *per se* on the injured spinal cord by using FoxJ1-CreER$^{T2}$-Rasless mice to block the cell cycle of ependymal cells. We found that with bOECs, the wound healing process was more efficient in FoxJ1-CreER$^{T2}$-Rasless mice, suggesting that bOEC transplantation *per se* benefits glial scar formation. Even though neuronal survival significantly decreased without the proliferation of ependymal cells, comparing FoxJ1-CreER$^{T2}$-Rasless mice to FoxJ1-CreER$^{T2}$-YFP mice, the number of survived neurons remained at the same level with bOEC transplantation from 2 weeks to 3 months after SCI, indicating that bOEC transplantation protects neurons from apoptosis to a certain degree. Moreover, qPCR data showed that there were no significant differences among the mRNA

level of axonal growth or inhibition molecules after SCI with or without transplantation in FoxJ1-CreER$^{T2}$-Rasless mice without proliferating ependymal cells. Therefore, these data suggest that the enrichment of microenvironment by bOEC transplantation is beneficial, but that ependymal cells are needed for the better effect.

It has been widely accepted that the regeneration of adult spinal cord is very limited and there is no adult neurogenesis in the spinal cord under physiology condition or after SCI (Barnabe-Heider et al., 2010; Sabelstrom et al., 2014; Sabelstrom et al., 2013). However, we unexpectedly discovered a few EdU+DCX+ cells two weeks after SCI with bOEC transplantation, and mature neurons co-labeled with EdU and NeuN were found three months after injury, suggesting that bOECs promotes adult neurogenesis after SCI. Interestingly, when we transplanted bOECs to FoxJ1-CreER$^{T2}$-Rasless mice where the cell cycle of ependymal cells was blocked, we did not find any newborn neurons. With co-transplantation of bOECs and ependymal cell-derived neurospheres, we did not find rescue effects of neurogenesis, suggesting that endogenous ependymal cells are necessary for the neurogenesis process promoted by bOEC transplantation. A recent study showed that using single nucleus sequencing (Div-Seq), rare neurogenesis was found in the adult spinal cord, and suggesting that the undiscovered spinal cord neurogenesis could be due to technical limitations in the past (Habib et al., 2016). Indeed, using immunohistochemistry, we failed to find any newborn neurons in both uninjured and injured spinal cords without bOEC transplantation. Therefore, our results suggest that together with ependymal cells, bOEC transplantation promotes adult neurogenesis to a detectable level by immunostaining, but more precise quantification needs to be performed in the future.

To further investigate the origin of newborn neurons and the progeny of endogenous progenitors upon bOEC transplantation, we used numerous transgenic mice: FoxJ1-CreER$^{T2}$-YFP, PDGFRα-CreER$^{T2}$-GFP, Connexin 30-CreER$^{T2}$-GFP, hGFAP-CreER$^{T2}$-YFP and GLAST-CreERT2-Tomato to fate map ependymal cells, OPCs, and subtypes of astrocytes (Ganat et al., 2006; Meletis et al., 2008; Mori et al., 2006; Rivers et al., 2008; Slezak et al., 2007). We found that the GLAST and Connexin 30 lineage cells give rise to DCX+EdU+Reporter+ or DCX+Reporter+ cells after two weeks of bOEC transplantation in the injured spinal cord, suggesting that some subtypes of astrocytes might be the origin of progenitors that give rise to newborn neurons. Long-term lineage tracing for these cells is ongoing, and we expect to see NeuN+EdU+Reporter+ cells from Connexin or GLAST lineages.

Furthermore, a recent study showed that a new technique, DroNc-Seq can perform sensitive, efficient and unbiased classification of cell types for charting systematic cell atlases (Habib et al., 2017). We used DroNc-Seq to analyze the single cell profile after SCI with and without bOEC transplantation. We found 16 subpopulations of neural cells with different gene expression profile, some of which are unreported cell types. Moreover, we also discovered significant alteration of gene expression at both cluster and single nucleus level comparing SCI animals with and without bOEC transplantation, indicating that bOEC

transplantation indeed has effects on the endogenous stem and progenitor cells. We are currently completing the analysis, and expect to provide an atlas of the alteration in all the endogenous neural cells after bOEC transplantation in the SCI animals.

Altogether, our study provides integrative insight into the effects of bOEC transplantation on endogenous progenitors. These data give clinical relevance and the mechanistic understanding, revealing that together with ependymal cells, bOEC transplantation promotes gliogenesis and neurogenesis, inhibits axonal growth inhibitors, and promotes neuronal survival after spinal cord injury.

# 5 CONCLUSIONS

The adult spinal cord has limited regenerative potential, resulting in poor recovery after spinal cord injury. Moreover, the development of the spinal cord is still not well studied, and the link between the development of spinal cord progenitors and adult spinal cord regeneration has been largely overlooked. Therefore, this thesis sheds some light on the regulation of spinal cord progenitor development, the different regeneration capacity after SCI during juvenile and adulthood, and the mechanisms behind clinical cell therapies for SCI.

In **Paper I**, we discovered that FoxJ1, a classical transcription factor involved in ciliogenesis, has broader functions in the regulation of both spinal cord development and the stem cell potential after SCI. We found that during embryonic development, FoxJ1 is transiently expressed in neuronal and glial progenitors, which will further give rise to subsets of interneurons and two subsets of astrocytes and all ependymal cells. FoxJ1 is required for the maintenance of stemness of the progenitors during development and the stem cell potential during adulthood. After SCI, FoxJ1 is required for the normal stem cell potential, proliferation and migration of ependymal cells for regeneration.

In **Paper II**, we observed that the stem cell potential is confined to ependymal cells since P10 in mice, and the potential of self-renewal and oligodendrocytic differentiation decreases over time. Juvenile ependymal cells are more highly activated after SCI than adult ones *in vitro*, but their contribution to the glial scar formation *in vivo* is lesion size- and age-dependent. The resident astrocytes and stromal derived pericytes show higher regenerative potential at the juvenile stage, while ependymal cells serve as a backup regeneration candidate after SCI.

In **Paper III**, we found that after SCI, bOEC transplantation increases the proliferation and self-renewal potential of ependymal cells both *in vivo* and *in vitro*. The transplantation of bOECs promotes higher astrocytic differentiation of ependymal cells but reduces the axonal growth inhibitors after SCI. The microenvironment of the injured spinal cord is enriched after bOEC transplantation regarding less axonal growth inhibitor, a higher level of neurotrophic factors and better neuronal survival. Unexpectedly, we found newly born neurons after SCI with bOEC transplantation, highly probably from endogenous astrocytes, challenging the current central stream theory that there is no neurogenesis after SCI. We also provided an integrative map of the cell profile of endogenous progenitors with DroNc-Seq technique, revealing that the alteration of gene expression after bOEC transplantation in SCI animals.

Altogether, this thesis gives new insights into the development and potential of spinal cord progenitors and stem cells and provides inspirations for future SCI therapeutic possibilities.

# 6 FUTURE PERSPECTIVES

## 6.1 FOXJ1 REGULATES SPINAL CORD DEVELOPMENT AND STEM CELL POTENTIAL AFTER SCI

Foxj1 is associated with the production and function of motile cilia at many sites (Blatt et al., 1999; Brody et al., 2000; Chen et al., 1998; Lim et al., 1997). Together with recent studies, our data also showed that FoxJ1 is also required for the CNS development and stem cell potential after SCI (Jacquet et al., 2011; Jacquet et al., 2009; Li et al., 2018; Shimada et al., 2017). However, the mechanistic study is needed to understand further how FoxJ1 regulates the maintenance of stemness of progenitors and stem cells. Some studies have shown that FoxJ1 is a target of Shh, Mcidas and GemC1 during CNS development (Cruz et al., 2010; Kyrousi et al., 2015; Kyrousi et al., 2016; Yu et al., 2008). A recent study showed that suppressing Notch leads to the upregulation of FoxJ1, which further promotes ependymal niche development (McClenahan et al., 2016). These studies, however, only provide insights how FoxJ1 is regulated as a downstream target. Therefore, further study on how FoxJ1 regulates other molecules and how these regulation effects on neural stem/progenitor cells during development and after SCI are needed.

We hypothesize that FoxJ1 plays a dual role in the spinal cord, as it maintains the stem cell potential during development and after SCI, but seems to inhibit the dedifferentiation of ependymal cells in adulthood. Indeed, even though the deletion of FoxJ1 leads to lower proliferation and pre-differentiation of progenitors during spinal cord development, as well as results in lower self-renewal potential of ependymal cells in adulthood, it is interesting to note that the lack of one copy of FoxJ1 postnatally results in high neuronal differentiation *in vitro* (Li et al., 2018/Paper I). We did not find any newborn neurons in heterozygous FoxJ1-Knockout mice after SCI (unpublished data), but whether a complete deletion of FoxJ1 after SCI in the adult can induce neurogenesis is not known. By using FoxJ1::EGFP mice, we found that ependymal cells lose FoxJ1 expression when dedifferentiating and migrating to the lesion after SCI *in vivo*, and that ependymal cells lose FoxJ1 expression when generating neurospheres and differentiate into astrocytes and oligodendrocytes *in vitro* (unpublished data). These data suggest that FoxJ1 might be an inhibitor for spontaneous dedifferentiation of ependymal cells. Therefore, whether FoxJ1 has dual functions during development and after SCI, and how these phenotypes are regulated will be an exciting topic in Developmental Biology and Regenerative Medicine.

To be able to investigate the questions above in the future, a more precise genetically engineered mouse model is needed. Indeed, the models to study FoxJ1 and ependymal cells are under controversy. The transgenic mouse models for lineage tracing in Paper I are mainly based on a human FoxJ1 promoter, which arouses the doubts and critics that the transgene may influence the effect of endogenous FoxJ1 expression (Ren et al., 2017). Even though in previous studies and Paper I, we have characterized the expression of FoxJ1 and the phenotype of ependymal cells are highly similar to WT mice during development, adulthood and after SCI, it will be less concerned by peers if a mouse model with

endogenous mouse FoxJ1 promoter can be developed (Barnabe-Heider et al., 2010; Li et al., 2018; Meletis et al., 2008). On the other hand, a knock-in mouse model with endogenous FoxJ1 promoter was recently developed, and researchers claimed that it is feasible as a lineage tracing tool to study FoxJ1 expressing cells (Muthusamy et al., 2014). However, despite the advantage of its endogenous promoter, a CreER locus was inserted into an exon of the FoxJ1 gene, resulting in the deletion of the FoxJ1 expression (Muthusamy et al., 2014). The phenotype of ependymal cells and other neural progenitors are thus influenced (Li et al., 2018/ Paper I). Moreover, as the CreER is knocked in and disrupt the endogenous FoxJ1 gene, the animals with lineage tracing possibility are FoxJ1+/- or FoxJ1-/- since E1. Therefore, a mouse model with an endogenous FoxJ1 promoter and flox loci for conditional FoxJ1 knockout will be beneficial to study FoxJ1.

## 6.2 JUVENILE AND ADULT EPENDYMAL CELLS HAVE DIFFERENT REGENERATIVE MECHANISMS

Previous studies provided that ependymal cells play a crucial role in glial scar formation and can be targeted as a non-invasive therapy for SCI (Barnabe-Heider et al., 2010; Sabelstrom et al., 2013). Our study expands the understanding of ependymal cells that juvenile ependymal cells have higher stem cell potential than adult ones but serve as a backup rescue player after SCI. We also found that juvenile spinal cord is more regenerative than adult (Li et al., 2016/Paper II). We performed dorsal funiculus lesion and dorsal hemisection lesion to the animals, both of which give clear observation possibilities to study different cell types. Even though these two models are not the perfect model to mimic clinical cases, compared to a contusion injury model, previous studies have provided other SCI models, such as lateral spinal cord lesion and crush injury, and show similar response of ependymal cells in dorsal injury models (Barnabe-Heider et al., 2010; Lacroix et al., 2014; Meletis et al., 2008; North et al., 2015). However, to be further study the application possibility of modulating ependymal cells, further characterization by using more clinically relevant SCI models is needed. Moreover, even though neurosphere-forming cells were found in the adult human spinal cord, there is no direct proof that whether these stem cells are originated from ependymal cells, and whether other cell types display stem cell potential after SCI in human (Dromard et al., 2008). To be able to move forward the ependymal cell-based therapy, more investigation on human tissue is needed.

The other perspective regarding modulating ependymal cells after SCI is whether we can rejuvenate the aged ependymal cells and progenitors for better regeneration. The mechanism behind the difference of regenerative potential comparing juvenile and adult stem cells and progenitors is not in-depth studied yet. However, several signaling pathways and molecules have been found to be associated with stem cell aging throughout different organs, such as FGF, mTOR, Wnt and TGF-β (Brack et al., 2007; Chakkalakal et al., 2012; Chen et al., 2009; Yousef et al., 2015). In the central nervous system, the system melieu has been shown to be important for aging and rejuvenating neural stem cells (Ruckh et al., 2012; Villeda et al., 2011). Furthermore, a recent study showed that microRNA derived

from stem/progenitor cells is capable to reserve aging in the brain (Zhang et al., 2017). Therefore, further studies on these molecules and microenvironment for ependymal cells after SCI will be beneficial for further modulating these stem cells.

## 6.3 BOEC TRANSPLANTATION EFFECTS ON THE REGENERATIVE POTENTIAL OF ENDOGENOUS STEM/PROGENITOR CELLS

Transplantation of OECs to injured spinal cord has been extensively used in SCI study, both in animals and human and achieve functional recovery, by modulating the glial scar and benefit axonal regeneration (Gomez et al., 2018). Our study provides insights into the transplantation effects from another perspective, showing that bOEC transplantation promotes the regenerative potential of endogenous ependymal cells, astrocytes, and stromal derived pericytes. While the manuscript is still incomplete, we will further address the question how the fate of endogenous stem cells and progenitors are affected after bOEC transplantation, by using different lineage tracing models to fate map ependymal cells, different subtypes of astrocytes and OPCs. Together with a newly developed single nucleus sequencing technique, DroNc-seq, the lineage tracing results and the RNA profile of single cells will provide an integrative map of the cell fates after bOEC transplantation treatment on SCI mice.

The adult spinal cord has been long seen as non-neurogenic, but we found that bOEC transplantation can unexpectedly promote adult neurogenesis after SCI, which was traditionally seen as impossible. To be able to understand further the adult neurogenesis process promoted by bOEC transplantation, we will perform another single nucleus sequencing, Div-Seq to study the subtype of newborn neurons. Taking advantage of the lineage tracing mouse lines, we will further trace the origin of the newborn neurons. Besides, we will perform electrophysiology on these newborn neurons for functional characterization.

Clinically, the patients who received autologous OEC transplantation obtained functional recovery, but the sacrifice of half of the olfactory bulb still causes concern of ethical issues and the risk of impairing smelling sensitivity (Tabakow et al., 2013; Tabakow et al., 2014) (Cell transplantation 2013). Analyzing and identifying the molecules secreted by bOECs will probably replace the whole bulb isolation with industrial synthesized factors, and decrease the complication of this therapy and increase the neurogenesis efficiency.

Altogether, this thesis gives new insights into the development and potential of spinal cord progenitors and stem cells, and provides inspirations for future SCI therapies.

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