Human and mouse spinal cord : a territory of diverse neural stem/progenitor cells, identification and functionality

Hussein Ghazale

THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

Ecole Doctorale Sciences Chimiques et Biologiques pour la Santé CBS2

U1051 - Institut Des Neurosciences De Montpellier

Human and mouse spinal cord: A territory of diverse neural stem/progenitor cells, identification and functionality

Présentée par Hussein GHAZALE

Le 12 Juin 2019

Sous la direction de Jean-Philippe HUGNOT
et Bernard ROTHHUT †

Devant le jury composé de

Mme. Julie Pannequin, DR2, CNRS IGF, Montpellier	Présidente du Jury
M. Nicolas Guerout, Maître de conférences, Fac de Médecine, Rouen	Rapporteur
M. Olivier Raineteau, DR2, INSERM U846, Lyon	Rapporteur
Mme. Emmanuelle HUILLARD, CR1, CNRS UMR7225, PARIS	Examinateur
M. Jean-Philippe Hugnot, PU, INSERM U1051, Montpellier	Directeur de These

UNIVERSITÉ DE MONTPELLIER

Dedicated

To my parents Fatima and Mohammad,

To my sisters and Brothers Samah, Ghaidaa, Ahmad, Hassan, and Rayan

And to my four angels Souma, Fatima, Zia, and Maya

Acknowledgments

It always seems impossible until it's done

First and foremost I want to thank my advisor Jean Philippe HUGNOT (JP). It has been an honor to be his Ph.D. student. He has guided me through performing the optimal experimental techniques. For that, I appreciate all his contributions, time, ideas, and funding to make my Ph.D. experience productive and stimulating. I would like to express my dearest gratitude to the AFM and all resources that contributed and fully funded my 4 years research program. An honorable mention goes to my co-director Bernard, who is no longer among us today. Thank you for everything and may your soul rest in peace.

The members of the Hugnot's group have contributed immensely to my personal and professional time at The INM. I would like to acknowledge honorary group members Philippe Geague and Chantal Ripoll the enthusiast, intense, and cooperative co-workers. Sophie, Nicolas, Valeria, Meera, Robert and Sophie muxel. It has been a great pleasure working and sharing with you my pipettes and tips. We spent amazing time next to each other on the bench and we shouldn't forget the unforgettable moments we shared.

Naurber Bakalara, Jack, Christine, Zahra, and Emilie thank you all for sharing your knowledge, findings, advice, and care. Christine you are special to me, thank you for your advice, and thank you for all the care you gave to me.

I want to share a million thanks with my lab seniors who introduced me to lab culture. You had led by example in every aspect of life. Safa, Soumaya, and Ali you are such good friends and mentors. You have contributed a lot in my administration and being the best translators. We have spent the most wonderful time working together in cell culture. Thank you for the crazy laughter's and making things way easier.

A big thank goes to the Institute members, and sure some speciel members who were more than colleagues, Jing, Alicia, Claire, Roxane, Alexandra, Jamal and Richard. Thank you for sharing with me love and care and thank you for your great support.

Nesrin, Salim, and Chamsa, the ballaleit all the way from Algeria. Thank you for being amazing traveling and food partners. You are all good hearted and you had a big part of my journey. Nesrin I shared with you the same office for three years, you were supportive to me in all the time. Chamsa the ballout of nesrin, such a nice guy with those nice blazers. Salim, such a great person with a great personality. You are a person who gives from his heart but never asks to be

paid back. I shared with you lot of crazy moments and funny time and lot of coffee breaks. Thank you all guys and I hope more of these days to come to keep out friendship fruitfull.

Shafika Assad, my BS or MS Doctor, I am grateful for giving me strength and confidence. Thank you for inducing me to embark on my journey. An honorable mention of Firas Kobeissy and Jihane Soueid my master 2 supervisors. Thank you for being next to me, teaching me, and supporting me. Working under your supervision, has opened a lot of doors to me.

University friends Aida, Yara, Pamela, Miriam and Georges, thank you for the amazing moments we spent together. Your presence on this day, would have had an added value. To my special university friends and more Aya, Jamal, Lama, Rami, Ismail, and Moustafayour friendship and support is priceless.

Naify my best friend at the Lebanese university, a great place full of hard times but amazing memories. We have been together through exams stressful times. Our long nights working at the lab in AUB along with Sarah. Thank you both for your calls and checkups. Despite of our busy schedules, you remained true to our friendship.

The Lebanese family of Montpellier;

To the Lebanese group, Adham my classmate, Amani my logical reference, Baraah my gym and crime partner, Catherine my funny girl, Fatima the chef of the group and the beautiful one, Maya the calm cute girl., hassan and hammoud the nice guys of the group. Thank you all for being with me and encouraging me. Thank you for all the nice time and the crazy moments we had. Thank you for all the dinners prepared by the chef. Thank you for your support, I love you all.

Those who touched my life in any way since we started PhD. Some special words of gratitude go to my friends who have always been a major source of support when things would get a bit discouraging. Jihad Karam, hope this Ramadan will not be tough for us w nshalla ykon syamak ma2boul. Diala Kantar, thank you for chopping the onions and for the French nationality. Amara ya amara, thank you for all the dinners you made “khsouse waet li ilna zmn msh 3amlin 23de”, and for being a great support in the airplane in our way back from Ghardaia. M3allem tannous, was really nice sharing with you Dabke in many occasions, sharing with you dinners, laughs and high voice, and watching all marvel movies together. The crazy William, I will be offering nice food in my defense I’m sure you will like it. Thank you for inviting me to the worst RAP party, a palce I never visit again, and sure you are the person who can never be

trusted. ZOUZOU al amir dash, too many things to thank you for, but mainly for the lahme b3ajeen, and for the wara2 el 3enab, sah keno mahro2en bas ktr tayben, mara tenye mnentebeh ma nehre2on. Les vaches joelle and jovon, the craziest, funniest and the weirdest girls I've ever met. Nassar Joelle issue 1 (TEZOOO) thank you for offering me your apartment after suffering for the first two days without "Zeit", I know it was crazy but it was fun. I know I scared you a lot that time but it was my favorite part. I can't forget the Chandelier choreography we designed, the Lez walks, and making fun of people, I know we were big EVILS. Jovon issue 2, since the first day you arrived you started making troubles because of your "casualism", but the part of your personality that I love the most. Thank you for being you, caring about us and taking care of us. Without you we could be dead or in a hospital or handicapped. I will never forget all your casual moments, and the laughs we shared because of you. I'm so grateful to pass those years with you and make fun of lot of people a reason why no one likes us. Too many memories to remember with you all guys and all are implemented in my memory. Who can forget the trips we had together the hotels we shared, the laughs till death, the food, les yeux nique, and others… We were together in all situations and at any time, and this was the most supportive wonderful thing we had in our hard time in France. Thank you guys for always being there for me, and the time we spent together is priceless and I will never forget.

Here a thank you would not be enough. Your support and love are bigger than everything I can describe. My back-bone and foundation. They are my parents who supported me with the first step I made till the day I stand here, and their support will never fade away. Thank you mom, dad, for showing faith in me and giving me liberty to choose what I desired. Never to mention, my siblings who never failed to act as my mentors, idols, educators, you name it. Their unconditional love forged the person I am today. Words will never be fair to describe your love and provision. I appreciate your late work hours to invest in me. I want to thank 4 little butterflies who melt my heart (Souma, Fatima, Zia, and Maya), Thank you for teaching me how to love and care. At the end, I would like to dedicate this thesis for you and for my big family in Lebanon. I don't imagine a life without their love and blessings.

Table of Content

List of Abbreviations

ALS	Amyotrophic Lateral Sclerosis
AIS	Axon Initial Segment
AP	Action Potential
ASCs	Adult Stem Cells
BDNF	Brain Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
CNP	2¢, 3¢-cyclic nucleotide 3¢-phosphodiesterase
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
COPs	Committed Oligodendrocyte Precursor cells
CSF	Cerebro-Spinal Fluid
CSF-N	CSF Contacting Neurons
DCX	Doublecourtin
ESCs	Embryonic Stem Cells
ECs	Endothelial Cells
EZ	Ependymal Zone
ERG	Ependymo-Radial Glia
GDNF	Glial Cell Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Aspartate Transporter
HFSCs	Hair Follicle Stem Cells
HGF	Hepatocyte Growth Factor
Iba1	Ionizing Calcium-Binding Adaptor Molecule 1
IGF-1	Insulin Growth Factor-1
iPSC	Induced Pluripotent Cells
ISCs	Intestinal stem cells
MAG	Myelin-Associated Glycoprotein
Map2	Microtubule Associated Protein 2
MFOL	Mature Myelinating Oligodendrocytes
MS	Multiple Sclerosis
MSCs	Mesenchymal stem cells

NeuN	Neuronal Nuclear Antigen
NFOs	Newly Formed Oligodendrocytes
NGF	Nerve Growth Factor
Nogo-A	Neurite Outgrowth Inhibitor A
NPCs	Neural Precursor Cells
NSCs	Neural Stem Cells
OLs	Oligodendrocytes
OLPs	Oligodendrocyte Progenitors
OMgp	Oligodendrocyte Myelin Glycoprotein
OPCs	Oligodendrocyte Progenitor Cells
PDGFRa	Platelet Derived Growth Factor Alpha
PNCs	Perineuronal Satellite Cells
PNS	Peripheral Nervous System
PSA-NCAM	Polysialated Form of Neuronal Cell Adhesion Molecule
RGLs	Radial Glia-Like Cells
SCI	Spinal Cord Injury
SGZ	Sub-Granular Zone
s-OLs	Satellite Oligodendrocytes
SPCs	Stem/Progenitors Cells
SVZ	Sub-Ventricular Zone
TACs	Transient Amplifying Cells
TBI	Traumatic Brain Injury
TNF α	Tumor Necrotic Factor
VEGF	Vascular Endothelial Growth Factor
VLMCs	Vascular and Leptomeningeal Cells

List of Figures and Tables

List of Scientific Papers

1. H Ghazale[*1], C Ripoll[*1], N Leventoux[1], D Mamaeva[1], S Azar[1], P Guigue[1], CF Calvo[2], JL Thomas[3], L Jacob[3,] S Meneceur[4], Y Lallemand[4], V Rigau[1,5], F Perrin[6], HN Noristani[7], B Rocamonde[3], E Huillard[3], L Bauchet[1,6,] JP Hugnot[1,7]. **The adult human and mouse spinal cord ependymal region maintain an embryonic-like dorsal-ventral regionalization with dorsal Msx1+ neural stem cells**

   *: co-first authors; Published

2. H Ghazale[1], C Ripoll[1], C Chalfouh[2], F Perrin[3], H Noristani[3], L Bauchet[1,4] , N Guerout[2], JP Hugnot[1,4] . **Spinal cord stem cell niche : from quiescence to activation after spinal cord injury.**

   In Preparation

3. H. Ghazale[1], P. Guigue[1], S. Benlefki[1], C. Raoul[1], P. Durbec[2], B. Rothhut[1], J. - P. Hugnot[1.] . **Peri-Neuronal Satellite Cells in the Central Nervous System: Isolation and Characterization.**

   In Preparation

Abstract

Over the last 10 years, JP Hugnot's lab has been focusing on the different pools of progenitors and stem cells found in the adult spinal cord both in human and mouse. This is important to conduct this kind of research as the spinal cord is affected by several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and traumatic lesions for which there is no cure. In anamniotes such as Zebrafish, the spinal cord can regenerate after lesion due to endogenous progenitors/stem cells activation. So by investigating the presence and properties of such cells in mammals especially human, one could possibly harness those cells toward regeneration including neurons. We conducted RNA profiling to compare human vs mouse stem cell niche and lesioned vs non lesioned mouse spinal cord stem cell niche. This niche is particularly interesting as in anamniotes, radial ependymoglia cells located in this region are multipotent and can generate new motoneurons after lesion. And similar, albeit non identical, cells are present in mouse. In mammals, after lesion, niche stem cells actively proliferate and migrate to generate mainly astrocytic cells and few oligodendrocytes which participate to the glial scar and regeneration by providing neurotrophic factors such as CNTF, HGF, and IGF-1. This niche contains at least 5 cell types and here a new dorsal cell type expressing Msx1 and Id4 transcription factors was identified. These results indicated that the adult spinal cord niche in mouse and human is a mosaic of cells with different developmental origin and maintaining high levels of neural developmental genes. Glial-neuronal interactions supporting and keeping neurons intact can influence neurodegenerative diseases. One of these glial cells is the satellite oligodendrocyte or so called perineuronal satellite cells (PNCs). PNCs are tightly associated to the soma of large neurons and widely spread in the grey matter of the CNS both cortex and spinal cord. However the cellular properties and functional roles of these unmyelinating oligodendrocytes are not yet discovered. In this study, nestin-GFP positive cells are associated to neurons immunostained for neuronal nuclear antigen in both cortex and spinal cord. We identified PNCs as CNPase positive cells that are neither oligodendrocyte progenitor cells (PDGFRa) nor myelinating oligodendrocytes (MBP). These data suggest that PNCs might affect neuronal survival as well as the myelination process in demyelinating conditions. Also it could be implicated in neurodegenerative diseases such as multiple sclerosis and amyotrophic lateral sclerosis due to their interaction with motor neurons.

Résumé

Au cours des 10 dernières années, l'équipe de recherche de JP Hugnot s'est focalisée sur la caractérisation des pools de cellules progénitrices et de cellules souches de la moelle épinière adulte, chez l'homme comme chez la souris. Cet axe de recherche est essentiel dans l'objectif de proposer un traitement dans le cas de nombreuses maladies neurodégénératives, comme la sclérose latérale amyotrophique (SLA), ou encore dans le cas de lésions traumatiques. Actuellement, il n'existe aucun traitement curatif. En revanche, chez des animaux comme le poisson zèbre, la moelle épinière peut se régénérer après une lésion grâce à l'activation de progéniteurs / cellules souches endogènes. Chez les amniotes, cette niche est particulièrement intéressante dans la mesure où, les cellules de l'épendymoglie radiale situées dans cette région sont multipotentes et peuvent générer de nouveaux motoneurones après une lésion. Ainsi, en recherchant la présence et les propriétés de telles cellules chez les mammifères, en particulier chez l'homme, on pourrait exploiter ces cellules afin d'initier une régénération, et la formation de nouveaux neurones. Nous savons que chez les mammifères, après la lésion, des cellules de la niche prolifèrent et migrent activement pour générer principalement des cellules astrocytaires (peu d'oligodendrocytes) qui participent à la cicatrice gliale et à la régénération en fournissant un facteurs neurotrophique tel que le CNTF, le HGF et l'IGF-1. Nous avons procédé au profilage des ARN en comparant 1-ceux issus de la niche de cellules souches humaine et de souris, 2- ceux issus de la niche de cellules souches de souris de la moelle épinière lésée et non lésée. Nous avons identifié au moins 5 types cellulaires différents et un nouveau type de cellules dorsales exprimant les facteurs de transcription Msx1 et Id4. Ces résultats indiquent que la niche de la moelle épinière adulte chez la Souris comme chez l'homme est une mosaïque de cellules ayant différentes origines développementales et conservant des niveaux élevés de gènes de développement neural. Par ailleurs, si les interactions cellules gliales - cellules neuronales participent au maintien des neurones intacts elles peuvent aussi influer sur les maladies neurodégénératives. L'une de ces cellules gliales est l'oligodendrocyte satellite ou cellules satellites périneuronales (PNC). Les PNC sont étroitement associés au soma de gros neurones et largement répandus dans la substance grise du cortex et de la moelle épinière. Cependant, les propriétés cellulaires et les rôles fonctionnels de ces oligodendrocytes non myélinisants n'ont pas encore été découverts. Dans cette étude, les cellules positives à la nestine-GFP sont associées à des neurones identifiés par l'antigène nucléaire neuronal NeuN dans le cortex et la moelle épinière. Nous avons identifié les PNC comme étant des cellules positives pour la

CNPase qui ne sont ni des cellules progénitrices d'oligodendrocytes (PDGFRa) ni des oligodendrocytes myélinisants (MBP). Ces données suggèrent que les PNC pourraient affecter la survie neuronale, mais également pourraient être impliquées dans le processus de myélinisation. Ainsi, en raison de leurs interactions avec les motoneurones, les PNC pourraient être impliquées dans des maladies neurodégénératives telles que la sclérose en plaques et la sclérose latérale amyotrophique.

INTRODUCTION

1. Chapter I: The human and mouse central nervous system

1.1 Components of the central nervous system

The central nervous system (CNS) is responsible for integrating sensory information and responding by motor and cognitive behaviors accordingly. It consists of two main components: **brain** and **spinal cord**. The brain is encased in the skull, and protected by the cranium. The spinal cord is continuous with the brain, and is protected by the vertebrae.

1.1.1 The Brain

The mammalian brain is one of the most complicated structures in living systems, which consists of more than 100 billion neurons in the adult brain [1]. Each neuron communicate with other neurons through synapsis. This structure originates from a simple neural tube, followed by various differentiation processes [2]. The brain is usually considered to have seven basic parts; the most obvious anatomical structures are the prominent cerebral hemispheres. In mammals, the cerebral hemispheres (the outermost portions of which are continuous, highly folded sheets of cortex) are divided into four lobes (Figure 1). Brain have various functions from sleep arousal to body homeostasis, learning and memory, and finally body motor coordination. In another word, physiologically, the brain exert a centralized control over the other organs of the body [1], [2]. It acts on the rest of the body both by generating patterns of muscle activity and by driving the secretion of hormones. This centralized control allows rapid and coordinated responses to changes in the environment. Some basic types of responsiveness such as reflexes can be mediated by the spinal cord or peripheral ganglia, but sophisticated purposeful control of behavior based on complex sensory input requires the information integrating capabilities of a centralized brain.

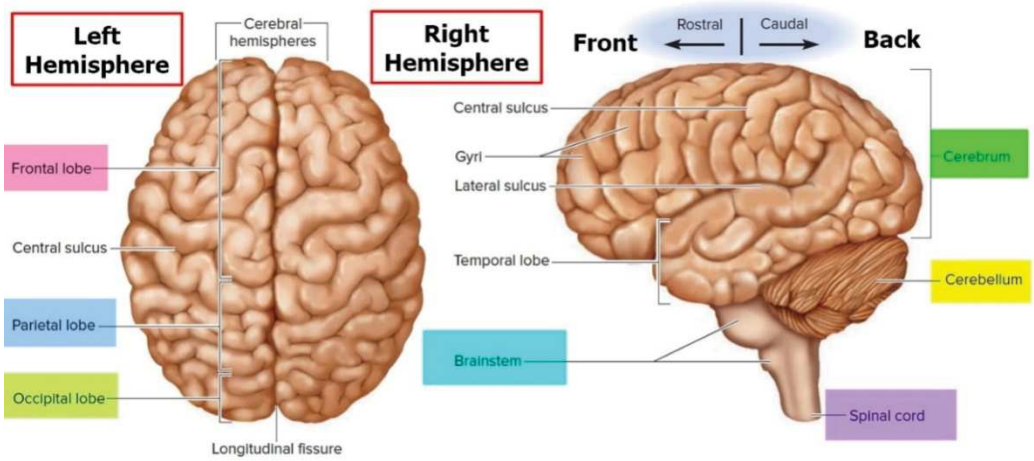


Figure 1: Anatomy of the brain.
The architecture and the outer anatomical structure of the human brain [3].

1.1.2 The Spinal cord

The spinal cord is made from part of the neural tube during development. There are four stages of the spinal cord that arises from the neural tube: The neural plate, neural fold, neural tube, and the spinal cord. Neural differentiation occurs within the spinal cord portion of the tube. The **spinal cord** is a long, thin, tubular bundle of nervous tissue and support cells that extends from the medulla oblongata in the brainstem to the lumbar region of the vertebral column [4]. In humans, the spinal cord begins at the occipital bone where it passes through the foramen magnum, and meets and enters the spinal canal at the beginning of the cervical vertebrae [5]. The enclosing bony vertebral column protects the relatively shorter spinal cord. The outer layer of the spinal cord consists of white matter, i.e., myelin-sheathed nerve fibers. These are bundled in to specialized tracts that conduct impulses triggered by pressure, pain, heat, and other sensory stimuli or conduct motor impulses activating muscles and glands. The inner layer, or gray matter, has a butterfly-shaped cross-section and is mainly composed of nerve cell bodies. Within the gray matter, running the length of the cord and extending into the brain, lies the central canal through which the cerebral spinal fluid (CSF) circulates [3] (Figure 2). The spinal cord functions primarily in the transmission of nerve signals from the motor cortex to the body, and from the afferent fibers of the sensory neurons to the sensory cortex. It is also a center for coordinating many reflexes and contains reflex arcs that can independently control reflexes and central pattern generators. The spinal cord is the main pathway for information connecting the brain and peripheral nervous system (PNS).

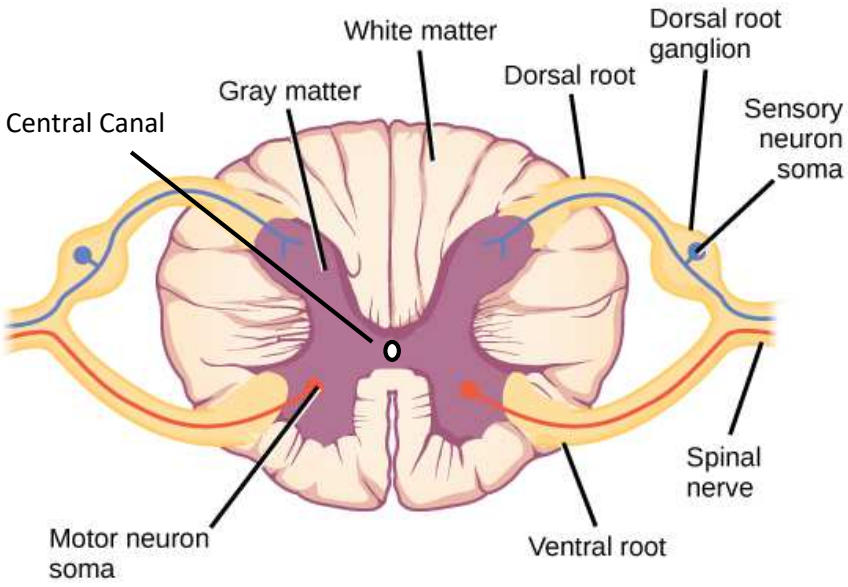


Figure 2: Anatomy of the spinal cord.
Cross section showing the anatomy of the spinal cord [3].

1.1.3 Human vs. mouse CNS

Pathologies affecting the CNS have diverse origins and forms, starting with Alzheimer's disease affecting the brain and multiple sclerosis (MS) affecting the spinal cord. In many instances, there is limited knowledge about the actual pathophysiology of the disease, about the succession of neurotoxic events following a damage. These issues make it difficult to assess accurately the prognosis of the pathology and the efficacy of treatments [6]. From this point, studies were conducted on animal models that help understand the disease, its evolution, and to develop novel therapeutic strategies. Past animal studies, primarily in rodents (mice and rats), have shown that development of distinct areas of the cortex is highly similar to that of the developing human cortex. For that reason, rodents were the best and the most used model among the mammals, due its availability, were easy to handle, and mainly because they share a similar CNS with humans with few differences in the structural organization. Those species share similar physiology, for instance, the connections between the striatum, subtantia nigra, and the cortex, responsible for Parkinson's disease (PK) in humans is more or less similar to that of the mouse [7]. Moreover, despite the differences in the size of spinal cord between human and rodents, they still share a similar architecture (figure 3). Also, parts of the white matter of the human spinal cord are almost larger than the entire diameter of the rat spinal cord, there is no significant difference in the migration capacity of oligodendrocyte progenitor cells (OPCs) to remyelinate axons in rats and humans [8].

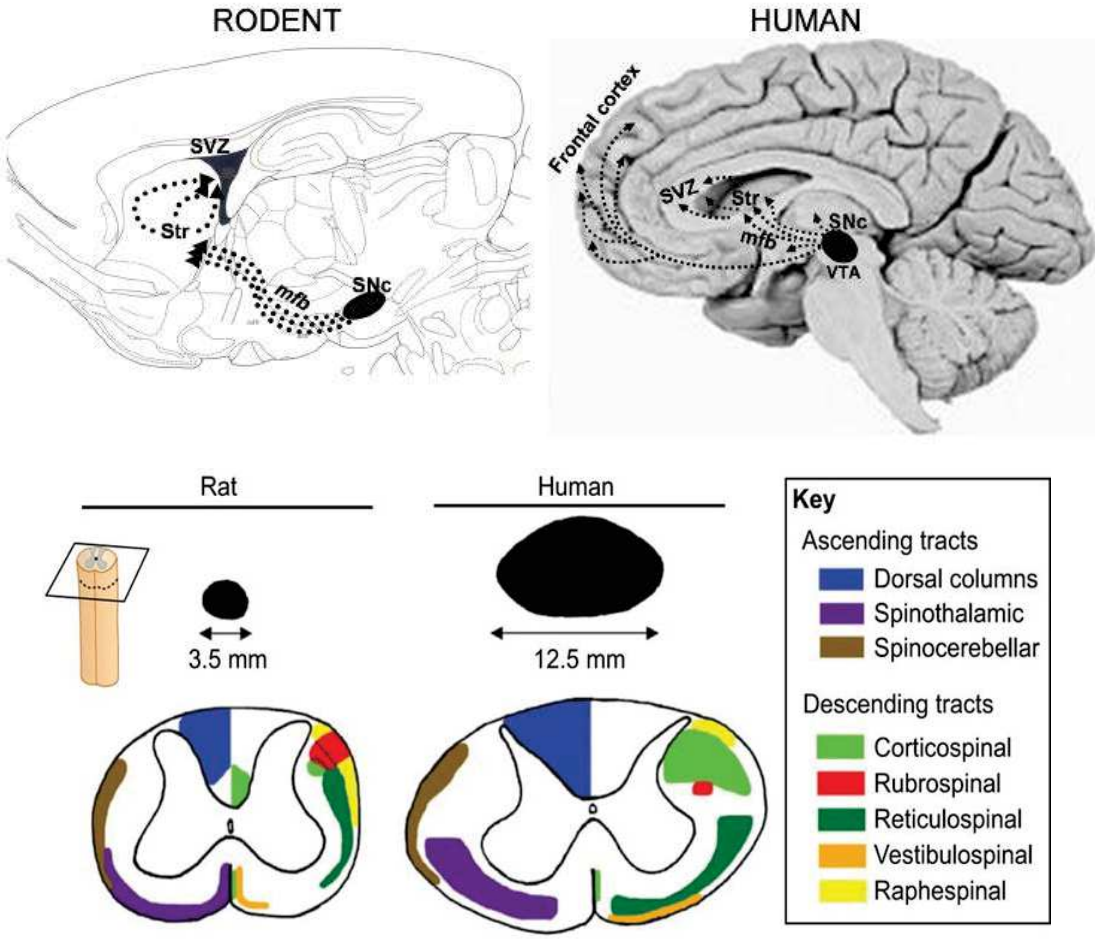


Figure 3: Human Vs rodent CNS.
Upper panel shows a Cross section of human and rodent showing similar brain connectivity [9]. Lower panel shows Color-coded representations of human and mouse spinal cords showing similar regional organization [10].

1.2 Cells of the central nervous System

At the microscopic scale, the CNS is made up of a complex and heterogeneous set of cells forming the neural tissue. The pioneering works of Ramon y Cajal at the end of the nineteenth century paved the way for the identification and precise characterization of these different cell types. Brain and spinal cord tissues are composed mainly of neuronal, glial, and endothelial cells [11], in which the neuronal function is highly dependent on glial cell physiology (Figure 3) [12].

1.2.1 Neurons

Neurons are the smallest units of information processing in the CNS. They are electrically excitable cells that receive, process and transmit information by electro-chemical signaling [11].The neurons then will generate what is called nerve impulse or action potential (AP) at the cell body, dendrite and then migrate along the axon. Then this AP will propagate via synaptic communication with the dendrites of adjacent neurons. Neurons are characterized by expressing markers that can be used to target neurons at different developmental stage and at different conditions. Neuronal nuclear antigen (NeuN) is a specific neuronal marker that is exclusively associated with the nervous tissue. This protein is localized in the nuclear and peri-nuclear cytoplasm of most of the neurons in the CNS. It emerges in the early embryogenesis in neuroblast and remains terminally in the differentiated neurons [13]. Doublecourtin (DCX) is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures. Neuronal precursor cells begin to express DCX while actively dividing. Due to the nearly exclusive expression of DCX in developing neurons, this protein has been used increasingly as a marker for neurogenesis, which is highly detected in the hippocampus and subventricular zone and olfactory bulb (sites of active neurogenesis). Microtubule associated protein 2 (Map2) is expressed mainly in neurons; their function is to stabilize microtubules in both dendrites and axons, and neuronal morphogenesis [14].

1.2.2 Glial cells

Glial cells were considered a minor cell type with limited function, but nowadays they are recognized as essential cell type for vital functions (neuronal homeostasis, maintenance of the blood-brain barrier and immune surveillance of the CNS). Glia cells play an important role in the development and the function of neural circuitry in the CNS, in which they constitute at least half the volume of the human brain and spinal cord [12]. There are five different types of glial cells:

1.2.2.1 Astrocytes

Astrocytes have a star shaped morphology as described early in 1893 by Mihály Lenhossék. Astrocytes constitute nearly half of the cells of the CNS, so they are involved in a wide range of CNS diseases. They are found in both gray and white matter [12]. Their functions are very diverse in which astrocytes control levels of some ions and neurotransmitters [15]. In this way, they communicate together via calcium waves and gliotransmitters, they intervene in

remodeling the development and the function of synapses, preserve the integrity of BBB, and support neuronal survival. It is also found that astrocytes play a major role in brain and spinal cord plasticity [12]. Moreover, they contribute to the formation of glial scars after CNS injury. Astrocytes can be detected by the glial fibrillary acidic protein (GFAP), a major intermediate filament protein expressed in astrocytes and neural stem cells. It's normally present at low levels in the cerebrospinal fluid (CSF), reflecting base line levels of astrocyte death. However, its level rises after TBI and continues to increase few days post-trauma [16].

1.2.2.2 Oligodendrocytes

These are small cells with multiple extensions and ramifications as described in 1928 by Del Rio Hortega. They are distributed in the white and gray matter of the CNS. Oligodendrocyte lineage cells pass through multiple differentiation process before the full maturation stage. Oligodendrocytes ensheath axons in the CNS to provide rapid conduction and metabolic support [17]. Over the past years it was thought that oligodendrocyte (OL) lineage cells are morphologically a heterogeneous population of cells and pass through three differential stages and generating three cell lineages [18]. But the advanced techniques proved that, OLs are morphologically and functionally diverse and pass through several differential processes and generate multiple cell types and subtypes [19]. Marques et al, analyzed 5072 transcriptomes of single cells isolated from various brain regions of juvenile and adult mouse, in which all the analyzed cells expressed markers from the oligodendrocyte lineage. Clustering methods led them to identify 13 distinct cell populations, where the differentiation path connecting OPCs and myelinating OLs is narrow that are diversified into six mature states [20]. The developmental progression and profile expression is summarized in figure 4, among these cell states there are:

- Oligodendrocyte progenitor cells also known as nerve/glial antigen 2 (NG2) glia or polydendrocytes [21]. They are widely distributed in the gray matter of the CNS and they are the glial stem cells that constitute a major glial population in the developing as well as mature CNS. These cells are able to proliferate and are multipotent. In particular, they act as a reservoir to ensure and maintain adult myelination in the white matter, and could play an important role in situations pathological [22]. Beside their well-established oligodendrocyte progenitor role, OPCs have been shown to actively participate in CNS signaling [23]. Early evidence showed that OPCs could generate

neurons, astrocytes, and Schwan cells when residing outside the neocortex. But recently it has been shown that NG2 expression commits the cells towards OLs [24]. OPCs are characterized by the specific expression of platelet derived growth factor alpha (PDGFR alpha), and NG2 (Cspg4). Several genes (Fabp1 and Tmem100) expressed by OPCs are previously described in astrocytes and radial glia consistent with the idea that OPCs originated from from glia-like cells, as well as their ability to generate astrocytes in response to injury [19]. OPC differentiation is triggered by neuronal chemical signals generating newly formed oligodendrocytes (NFO) that are no longer expressing the OPC specific markers.

- Different from the OPCs there are the differentiation-committed oligodendrocyte precursor cells (COPs), they lacked the expression of PDGFRα and Cspg4, but expressed Neu4 and genes keeping OLs in undifferentiated state (Sox6, BMP4, and Gpr17). Unlike OPCs, COPs express low levels of cell cycle genes, but the highly express genes involved in migration (Tns3 and Fyn) [20].

- Newly formed oligodendrocytes (NFOs), are generated from the differentiation of OPCs in response to differentiation signals from surrounding neurons. Several studies mentioned that, NFOs are genetically different from OPCs, and they are of two subtypes (NFOL1 and NFOL2). These cells expressed genes induced at early stages of differentiation (Tcf7l2 and Casr) while Gpr17 levels decreased. NFOLs are known to generate two cell types, myelinating and non myelinating oligodendrocytes. Early evidence suggested that NFOs or the non-myelinating OLs die within seven days after they born. But recent evidence showed that NFOs are maintained in the CNS as a reservoir of OLs in case of sudden injury and degeneration [20].

- Mature myelinating oligodendrocytes (MFOL), are first described by Del Rio Hortega in 1928. They are derived from the last differentiation step of OPCs. Thus, they differ from OPCs by the gene expression, protein profile, morphology, as well as the function. Marques et al subdivided this population into six subtypes (MFOL 1-6) that differed in gene expression, distribution, and function. MFOL1 and 2 are specialized by the expression of genes responsible for myelin formation such as myelin basic protein (MBP), proteolipid protein (Plp1), and other markers that can be targeted to study OLs. Due to their protein profile OLs are the myelinating cells in the CNS and MOLs1 to

MOLs6 expressed late oligodendrocyte differentiation genes (Klk6 and Apod) [20]. Their small branched morphology let the cells attach to the denuded axons of neurons through fine spines to generate myelin sheath in the white matter. So the function of OLs is to generate myelin around axons to accelerate the AP, and protect neurons from degeneration. While in the gray matter OLs are known to play a trophic role through the secretion of a bunch of neurotrophic factors such as, brain-derived neurotrophic factor (BDNF) , glial cell-derived neurotrophic factor (GDNF), and insulin growth factor 1 (IGF-1). Thereafter, in case of injury and degeneration, OLs proliferate extensively and migrate to provide support and protection of neurons at the site of injury. On the other hand, OLs are vulnerable to oxidative stress, the cause that could deplete OLs from the CNS in case of severe degeneration [25].

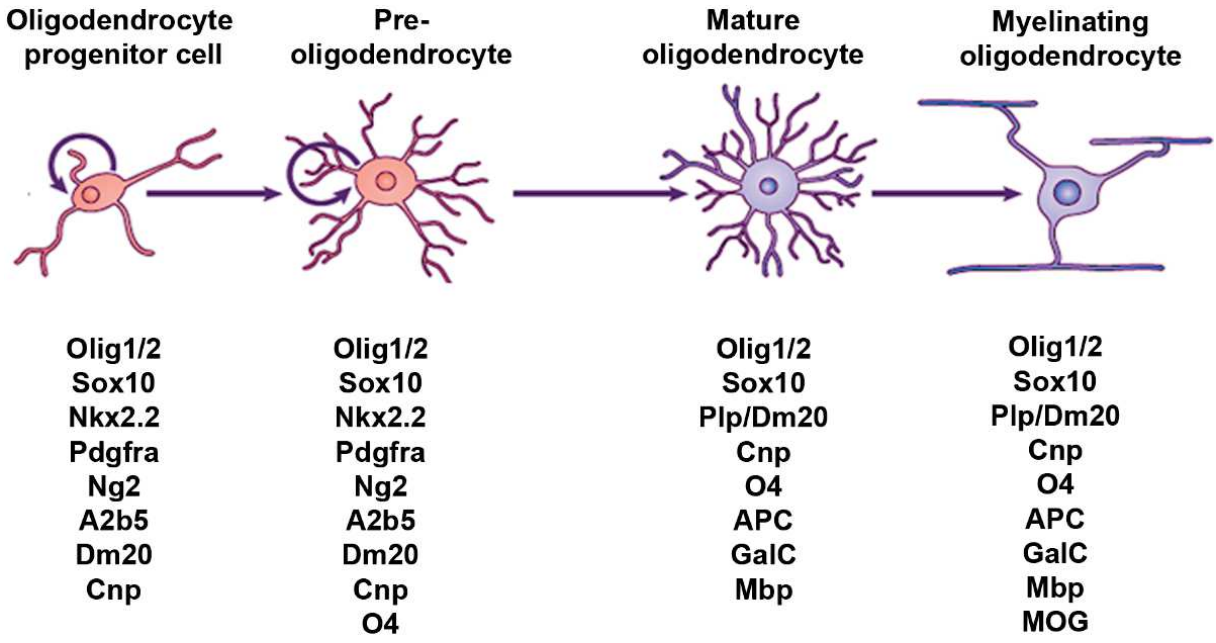


Figure 4: Schematic representation of the developmental stages of the OL lineage.
The morphological features of OPCs, pre-OLs, mature OLs and myelinating OLs and their antigenic profile are shown [26].

- **Peri-Neuronal satellite cells**

In addition to the six described oligodendrocyte cell lineages, several labs and our lab have identified a cell type that could belong to the oligodendrocyte lineage cells, but not described by Marques et al. Glial cells. They are attached or surrounded neuronal cell bodies including oligodendrocytes, astrocytes and microglia [27], [28]. Of these cells those are associated to the neuronal cell bodies in the cortex and referred to as perineuronal satellite cells (PNCs) or

satellite oligodendrocytes (s-OLs). Several evidence showed the increase of PNCs with age and reported that the highest population is derived from oligodendrocytes [27].

However, their cytochemical properties, cytological characteristics, and function are not well described yet. Takasaki et al, identified perineuronal oligodendrocytes as 2¢, 3¢-cyclic nucleotide 3¢-phosphodiesterase (CNP)-positive cells that are attached to neuronal soma expressing MAP2. The unmyelinating oligodendrocytes were immunonegative to specific glial markers for astrocytes (GFAP), microglia (Iba-1) and OPCs (NG2). However, almost all PNCs expressed glia-specific or glia-enriched metabolic enzymes, such as the creatine synthetic enzyme S-adenosylmethionine: guanidinoacetate N-methyltransferase and l-serine biosynthetic enzyme, and 3-phosphoglycerate dehydrogenase. Regarding molecules contributing to the glutamate–glutamine cycle, none of the PNCs expressed the plasmalemmal glutamate transporters GLAST and GLT-1, where nearly half of the perineuronal oligodendrocytes expressed glutamine synthetase. Cytologically, PNCs were distributed in deep cortical layers, mainly layers IV–VI, and characterized by the direct and tight attachment to neuronal cell bodies, inducing a concave shape in the contacted neurons (figure1 A, B). PNCs were more attached to glutamatergic principal neurons than to GABAergic interneurons, and this was evident at postnatal day 14, the day of cerebral cortex development and maturation. These cytochemical and cytological properties suggest that perineuronal oligodendrocytes are so differentiated as to fulfill metabolic support to the associating principal cortical neurons, rather than to regulate their synaptic transmission [29].

Following the identification of cytochemical properties, cytological characteristics, it was interesting to identify the genetic signature of perineuronal oligodendrocytes to reveal their unique phenotype. The study provided a genetic framework for a functional investigation of pN-OLGs. They identified oligodendrocyte transmembrane protein (OTMP) [30], a novel member of the glutamate-binding protein subfamily as the representative marker that highlights PNCs. They confirmed that in vitro, the immuno-purified polyclonal Ab, OTMP, co-localizes with mAb A2B5 on the surface of cultured live rat Oligodendrocyte progenitors (OLPs), but not with O4+ committed OLGs. The OTMP marker doesn't recognize neurons, differentiated OLGs, perineuronal astrocytes, and perineuronal microglial cells, thus OTMP Ab is a selective marker for pN-OLGs. Since TFs are the ultimate determinants of cell identity, they provided a comparative analysis of the A2B5+/OTMP+ transcriptome database with that of the A2B5+ progenitor or the committed O4+ OLGs. Interestingly, the transcription factors (TFs) are separated into two main categories.

The first set of assembled transcription factors includes DLX1 / 2, OLIG1, OLIG2, SOX10 and ASCL1, are known to play critical roles in the acquisition of the myelinating fate. However, these TFs stand behind the processes that drive OLPs toward myelin-forming OLGs – revealing the switch from the A2B5+ progenitor to the A2B5+/OTMP+ cells, but undergo drastic changes when acquiring O4. While the Olig2 expression stays invariant in the three cell types, the high expression of Dlx1 transcripts in A2B5+/OTMP+ cells predicts its role as an inhibitor of the myelinating phenotype. Also there is an increased expression of both ASCL1 and PAX6 in A2B5+/OTMP+ than in O4+ cells. In contrast, OLIG1 and SOX10, whose transcripts are increased in O4+ cells confined to their accepted role in determining the myelinating phenotype [31], [32].

Considering the second set of transcription factors, it includes PEA3 (aka ETV4), LHX2 and OTX2 that are not previously linked to the OLG lineage, but known to be essential for motor neurons and Schwann cells. The PEA3 group (ERM, PEA3 and ETV1) is a member of the Ets domain of transcriptional regulators expressed in specific motor neuron pool. PEA3 controls central position and terminal arborization – a crucial step in the assembly of neuronal circuits. The high expression of this TF in in A2B5+/OTMP+ compared with O4+ cells suggest that PEA3 contribute to the non-myelinating phenotype. LHX2, and Otx2 has as high presence in A2B5+/OTMP+ cells and have important functions during development [32], [33].

Furthermore, the molecular mechanisms and signaling pathways that play crucial roles in patterning the CNS, and regulate the cellular processes that guide an A2B5 precursor towards either a non-myelinating (A2B5+/OTMP+) or a myelinating (O4) have been identified. The major pathways that contribute to oligodendrogenesis are sonic hedgehog (Shh) and Wingless (Wnt). As provided previously, Shh high expression is required for the earliest markers for OLPs, Pdgfrα, however this marker was higher in A2B5+/OTMP+ relative to A2B5+ OLPs. In addition to Pdgfrα, the cells transcribe Pdgfrβ, and the growth factor Pdgfc – a high-affinity ligand for PDGFRαβ and a strong mitogen. This combination and hetero-dimerization of transcripts silence the response of A2B5+/OTMP+ cells to PDGFAA – a ligand for the myelinating phenotype [32], [34].

Turning to Wnt, the Wnt / β-catenin pathway directs the onset of neurogenesis and gliogenesis [35], and blocks the differentiation of OLPs [36]. Fzd2 transcript is higher in A2B5+/OTMP+ than in O4+ cells, suggesting that Wnt signaling maybe critical to the biology of PNCs. A downstream target of Wnt signaling is the NrCAM protein, an adhesion molecule that is found in neurons and Schwann cells, but not in myelinating OLGs. Further invesitgation is required to uncover the function of NrCAM in pN-OLGs.

Another pathway that contributes to scenario is Notch signaling that act in three distinguishing aspects in A2B5+/OTMP+ cells. First during neurogenesis there is a tight cross-regulation between Notch and the two transcription factors, ASCL1 and DLX1 / 2, which results in sequential specification of progenitors [37]. Second, there is upregulation of the Notch target Hes5, an inhibitor of myelin gene expression, and controls the two pivotal genes, Ascl1 and Sox10. PNCs have higher Hes5 and Ascl1 while Sox10 transcripts are expressed at a very low level, compared to O4+ cells. To conclude, HES5 is responsible for keeping myelin transcripts inactive in A2B5+/OTMP+ cells, thus its transcriptional code has to be tailored for the non-myelinating phenotype [32], [38]. Third, Notch1 drives the increased transcription of lipocalin-prostaglandin D2 synthase (L-PTGDS) in A2B5+/OTMP+ cells. In contrast to previous data that showed that Notch1 decrease PTGDS expression [39]. Indeed, the differentiation progression from A2B5+ progenitor into A2B5+/OTMP + cells results in an order of magnitude higher transcription of L-PTGDS as well as synthesis of the protein.

The previous data was followed by another study which focused on assessing and identifying the function of PNCs. The most important evidence that contribute to the regulation of neuronal activity is the tight organization of glial cells around neurons, called the glial syncytium (figure 6), suggesting essential functional roles of local glia arrangements [40], [41]. In the grey matter the most frequent glial type that contribute to this syncytium is OLs, in addition astrocytes and microglia, can be found in a satellite position around neurons [29], [42]. As mentioned previously, PNCs are primarily non-myelinating cells that cannot produce myelin [29], [32] thus contrasting the main function of myelinating OLs [17]. However, PNCs may provide metabolic support for neurons [29], protect neurons against apoptosis [43] or remyelinate axons following demyelinating injuries [44]. Interestingly, the presence of these cells directly at the base of the soma, close to the axon initial segment (AIS) (figure 1C), the action potential (AP) initiation site, enables them to influence AP firing [45]. Arne Battefeld et al investigated the functional and anatomical properties of s-OLs, by combining simultaneous whole-cell patch-clamp recordings and live-confocal imaging with post hoc immunofluorescence and electron microscopy of neuron–s-OL coupling in acute neocortical slices from adult mice. In contrast to previous studies, s-OLs wraps neurons with compact myelin and action potentials in the host neurons exhibit precisely timed Ba2+-sensitive K+ inward rectifying (Kir) currents in the s-OLs. Unexpectedly, the glial K+ inward current are not mediated by somatically expressed OL-specific Kir4.1 channels. Instead, the AP-evoked Kir currents are transmitted through gap–junction coupling with neighbouring OLs and astrocytes that form a syncytium around the pyramidal cell body. The repetitive high-frequency AP firing of pyramidal neurons is in part

controlled by the glial Kir that constrains the perisomatic [K+]o. In other words neurons with s-OLs showed a reduced probability for action potential burst firing during [K+]o elevations. These data show that s-OLs are integrated into a glial syncytium and perform multiple functional and anatomical roles, including spatially buffering K+ and myelinating axons within the perisomatic domain [46].

Little is known about the characteristics and function of PNCs, and most of the data were conducted on newly born rats and mice brains. Further investigation is required on the adult stage of these species as well as human. It's also interesting to further confirm the presence of PNCs in the intact spinal cord and whether these cells are in a tight cross-talk with motor neurons. So part of my PhD focused on characterizing PNCs in the intact and degenerative CNS, and studying the in-vitro characteristics of these cells.

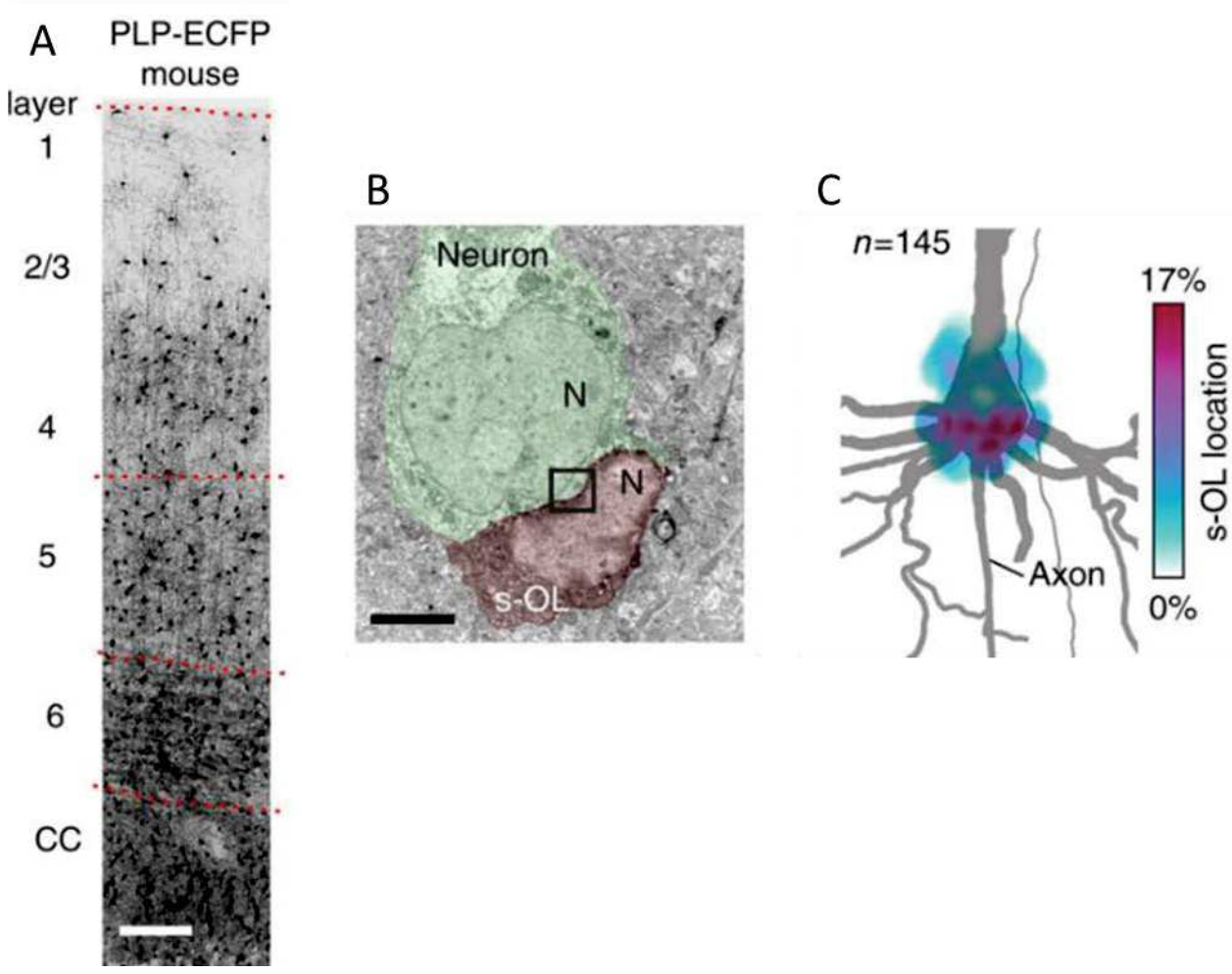


Figure 5: Identification and distribution of s-OLs around pyramidal neurons.
(A) Overview image showing the distribution of oligodendrocytes in the somatosensory neocortex the transgenic PLP-ECFP mouse. **(B)** Electron microscopic image of s-OL (red pseudo-color) in a tight association with neuron cell body (green pseudo-color). **(C)** Heat-map indicating the location probability of 145 s-OLs in relation to the soma of an example L5 neuron (grey) [46].

1.2.2.3 Microglial cells

They represent the resident macrophages, a cell type of the immune system, that constitutes 10% of CNS glia [47] . These small mobile cells are derived from myeloid progenitor cells that invade neonatal brain. Microglia are located in the parenchyma tissue of the brain and spinal cord. Like astrocytes, their function is not well known whether they are helpful or harmful [12]. They have a phagocytic ability, as well as, they secrete cytokines such as tumor necrotic factor alpha (TNF α), that can weaken the BBB integrity. Both in vivo and in vitro studies showed that microglia control normal function and plasticity of neural circuits of the brain [48]. One more important role of microglia is that they modulate synaptic activity, as well as, they selectively eliminate in appropriate synaptic connections during development of neural circuits [49]. Ionizing calcium-binding adaptor molecule 1 **(Iba1)** is a protein that is specifically expressed in macrophages/microglia and is up regulated during the activation of these cells [12].

1.2.3 Ependymocytes or ependymal cells

Ependyma is made up of ependymocytes, one of the four types of neuroglia in brain and spinal cord canal. They line the CSF-filled regions with a polarized ciliated simple columnar shape. It is involved in the production of CSF, act as a permeable barrier exchanges between the cerebral parenchyma and the CSF, and is shown to serve as a reservoir for neuroregeneration. Jonas Frisén and his colleagues provided evidence that ependymal cells act as reservoir cells in the forebrain, which can be activated after stroke and as in vivo and in vitro stem cells in the spinal cord. However, these cells did not self-renew and were subsequently depleted as they generated new neurons, thus failing to adopt the requirement for stem cells [50].

1.2.4 Endothelial cells

Endothelial cells (ECs) are the monolayer wall of the blood-brain barrier, and are in direct contact with the blood and lymph. They are the major cellular constituents of the CNS, in which they are involved in CNS development and function. Endothelial cells appear to guide developing axons (20). They also aid in providing trophic support as well as differentiation signals to neurons and stem cells (21). Finally they provide a niche for neural stem cells (NSCs) [15].

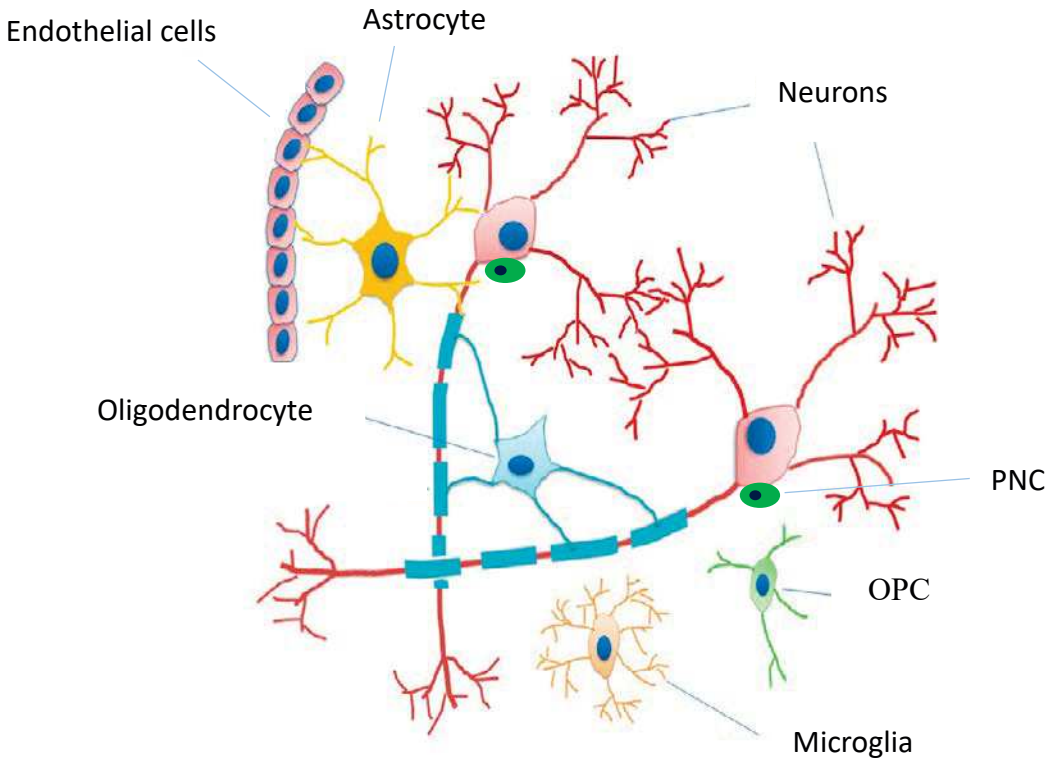


Figure 6: Schematic representation of glial-neuronal interaction.
Glial cells surround and interact directly with neurons and for the glial syncytium [51].

Chapter II: Stem Cells in their environment

2.1 Stem Cells outside the CNS

2.1.1 History of neural stem cells

Over the last decades till today, adult neurogenesis was a big debate among scientists, whether adult CNS was able to generate new neurons or its inability to produce new neurons or regenerate damaged ones. In 1962 adult neurogenesis was first described by Altman, J. However, in 1992 NSC were first isolated from the adult mouse brain, in which Reynolds, B.A proved the existence of adult neurogenesis. Since that time was the «no neurogenesis » dogma disproven. Although the definition of stem cells has yet to be agreed, stem cell research has been intensified to better understand, as well as characterize stem cells. They are undifferentiated cells that have unlimited expansion capacity, to create progeny through self-renewal and differentiation processes [52]. The self-renewal state is the ability to go through numerous cycles of cell divisions while maintaining undifferentiated state. Whereas the differentiated characteristic, which is known as stem cell potency, is the capacity to differentiate into specialized cell types that form the different tissues and organs of the body. These cells are present in embryonic tissues as well as postnatal and adult tissues. They multiply through either symmetric or asymmetric division to produce a pool of stem cells as well as differentiated cell types, to replenish dying cells and to regenerate damaged tissues [53]**.**

2.1.2 Characteristics and types of stem cells

CNS stem cells are defined as multipotent, self-renewing cells, which can differentiate into specific cellular type. Such cells exist transiently during fetal development but it was also confirmed that they exist in the adult mammalian CNS and maintained throughout life. They are of several types and have various properties. **Neural stem cells** (NSCs) are of different types and are used widely. In the early 90s several groups reported the isolation of NSCs from fetal and adult CNS.

2.1.2.1 Embryonic stem cells (ESCs)

Are pluripotent stem cells that have indefinite capacity of self-renewal and differentiation into cells of all three germ layers (figure 7). They are the ideal source for neural transplantation because of their high plasticity as they undergo both symmetrical and asymmetrical divisions. For instance, transplantation into damaged brain, ESCs can differentiate, migrate, and make

innervations. Thus ESCs derived from human or mice fetal CNS have been reported as a source of cell transplantation for injury and degenerative diseases in animal models. So transplanting either human embryonic stem cells (hESCs) or NSCs derived from mice fetal brain into the mice injured brain (at site of injury), showed significant improvement in motor and spatial learning functions, as these cells differentiate into neurons and glial cells that aid in tissue repair. However, ESCs have the drawback of ethical issue are prone to transplant rejection and may create teratomas when administered in-vivo. Although recent studies have isolated ESCs from areas of active neurogenesis in the SVZ of lateral ventricle of mice, they can also be harvested from the ganglionic eminences of mouse embryos at day 14 of gestation [54].

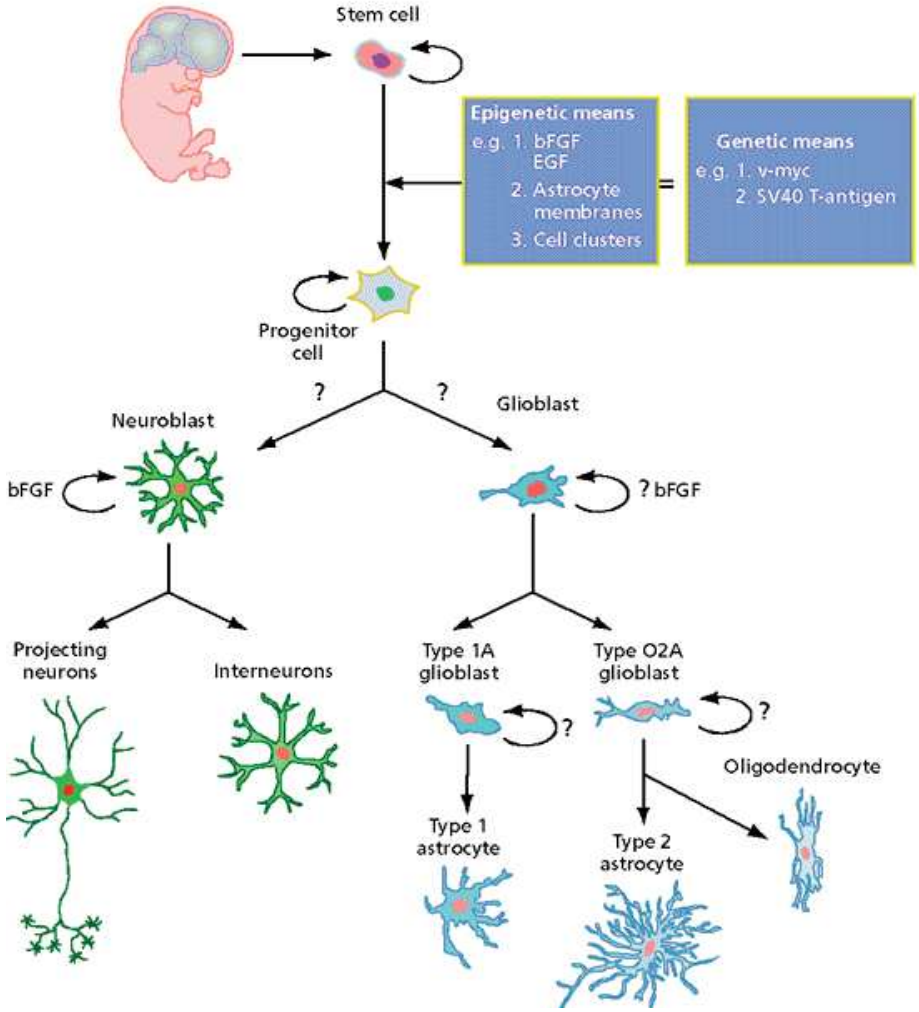


Figure 7: ESCs differentiation steps.
Differentiation of a single ESC into neurons and glial cells [55].

2.1.2.2 Adult stem cells (ASCs)

They are multipotent stem cells that can differentiate into unipotent cells of the residing tissue, generally for the purpose of repair. Unlike ESCs, ASCs have a decreased potential to self-renew and normally differentiate through only one lineage. Their ability to differentiate into neuronal cells demonstrated a possible therapeutic avenue for CNS diseases and injury treatment. ASCs are harvested from rodent adult brain and spinal cord where they are confined to the SVZ surrounding the lateral ventricle and central canal respectively (figure 8). When transplanted into the rodent injured CNS at the injury site, ASCs showed survival capacity for an extended period in the injured CNS. Moreover these cells migrate away from the injury site into surrounding areas, where they express markers of mature glial cells (astrocytes and oligodendrocytes). This cell property demonstrates that ASCs became region-specific functional cells. In addition to rat ASCs, human adult stem cells (hASCs) can be harvested from the same regions. Injection of cells of either source may restore the anatomy and function of injured areas [56], [57].

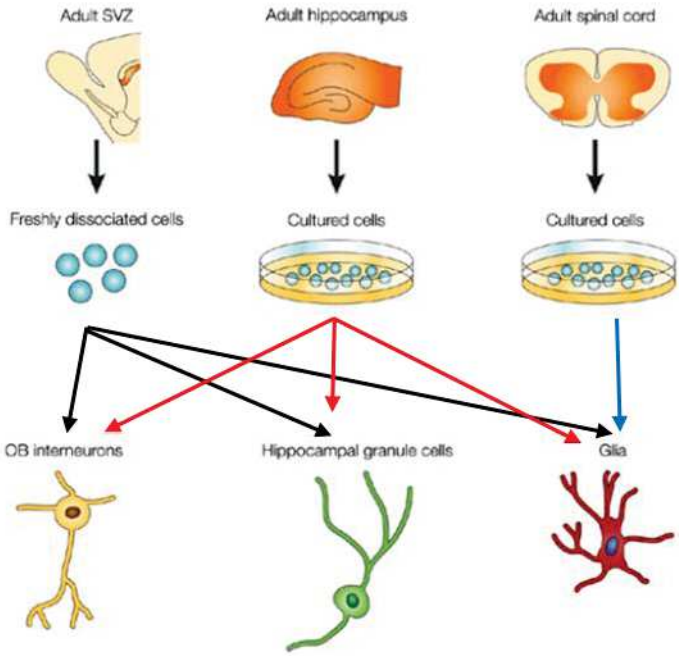


Figure 8: ASCs differentiation steps.
Differentiation of ASCs that are isolated from various CNS regions [58].

2.1.2.3 Mesenchymal stem cells (MSCs)

They are adult multipotent stem cells that are derived from non-neural tissue such as bone marrow (figure 9). These cells have both the ability to self-renew and the ability to differentiate down to multiple cell lineages, so they are considered to have potential therapeutic benefits in different neurological injuries. So these cells are harvested from either human or rodent models where they showed capacity of neuro-repair and neuroprotection. When they are administered intravenously or injected directly into the site of injury, MSCs are migrated away from the lesion and subsequently survive, and can differentiate into neurons and astrocytes, leading to injury repair and enhanced motor function [59], [60].

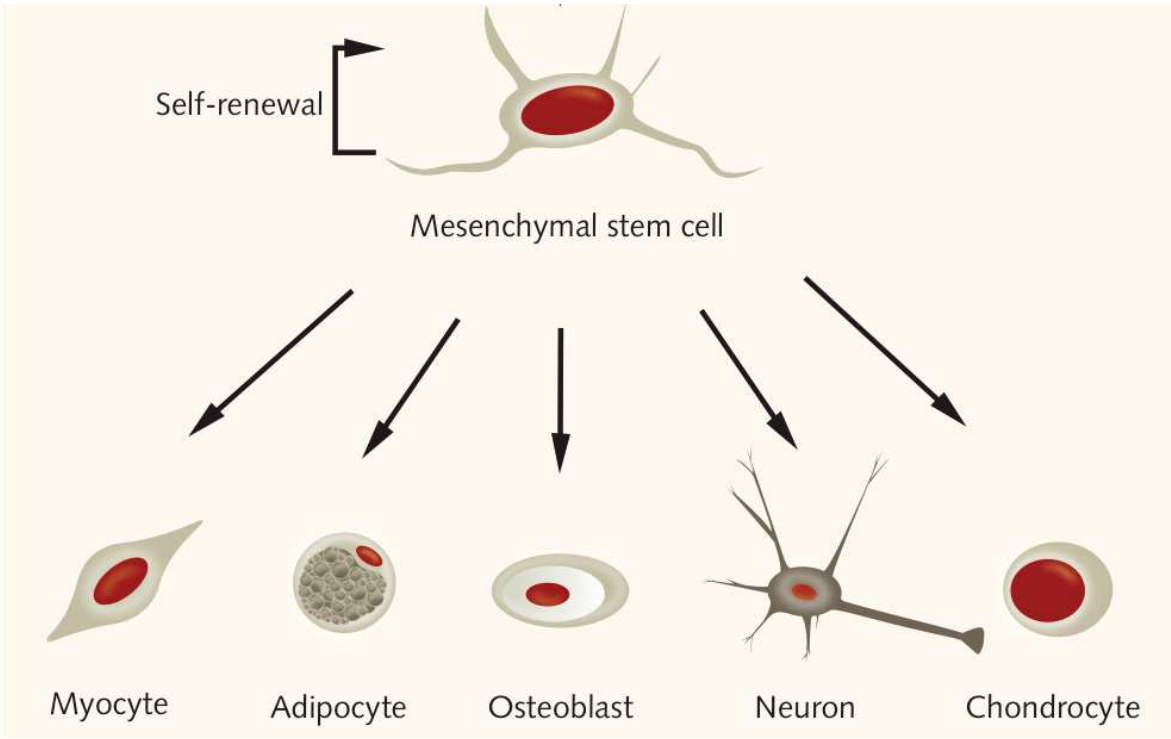


Figure 9: MSCs differentiation steps.
Self-renewal and differentiation capacity of mesenchymal stem cells [61].

2.1.3 Medical use

Many experimental attempts and trials for treating neurodegenerative diseases and CNS injury focus on neuroprotection during the intense period after injury. Despite the fact that this methodology holds positive outcomes, numerous individuals might either miss the little treatment window or may not completely profit by it. Rehabilitative treatments and medications that act on the post-acute period provide greater chance, therefore a long-term strategy may be required. Thus NSCs of both origins (embryonic and adult) represent a candidate to be used in the medical field as a long term treatment.

2.1.3.1 Neural stem cells in degenerative diseases

Studies have suggested that NSCs and neural precursor cells (NPCs) play a critical role in the onset and progression of various neurological diseases. NPCs dysfunction is highly associated with Parkinson's diseases (PD), Alzheimer's disease (AD), and Amyotrophic lateral sclerosis (ALS). However, functional NSCs and/or bioengineered NSCs is a recently discovered method to retain therapeutic potential. Recent work showed the ability to generate new neurons in both human and mouse to treat those diseases (figure 10). Currently, three different methods are being used to derive neurons from somatic cells. Somatic cells such as skin fibroblasts can be reprogrammed to become induced pluripotent cells (iPSC) and then differentiated into neurons and glia. They can also be trans-differentiated to become induced neural stem cells (iNSC), or directly trans-differentiated to become induced neurons (iN). The aim of these methods is to transplant those reprogramed cells or endogenously recruited or exogenously expanded cells in degenerative models. The newly generated neurons harbor several neuronal properties and have spontaneous activities. They carry sodium and potassium channels and are able to generate AP in response to stimulation. Thus after transplantation those neurons are functionally integrated and active, resulting in impeding disease progression and enhancing motor and behavioral improvements [62], [63].

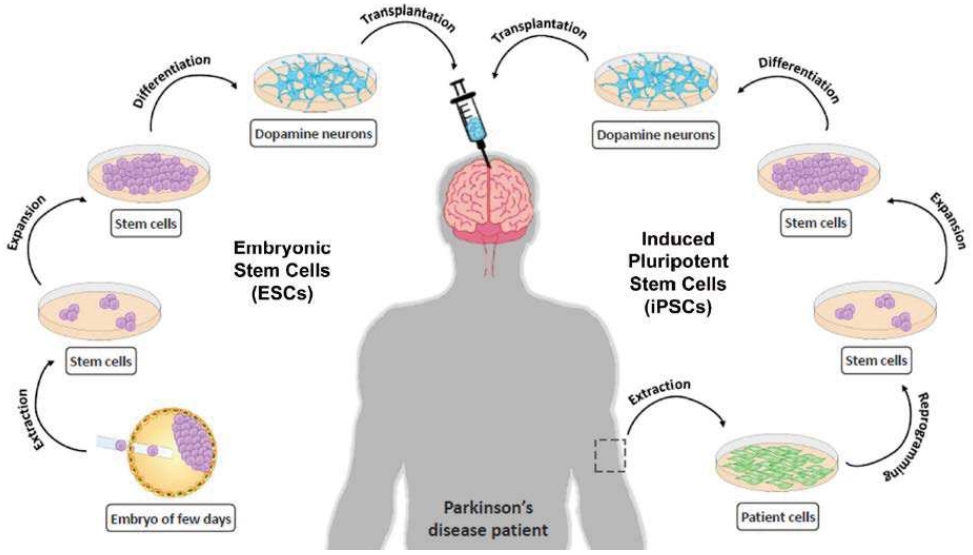


Figure 10: Treatment strategies.
Stem cells transplantation as a strategy in treating degenerative diseases (Raquel Coronel, 2015).

2.1.3.2 Neural stem cells in injury

CNS injury is associated with a wide range of post injury complications, where there are no FDA-approved drugs to treat traumatic brain injury (TBI) or spinal cord injury (SCI). Several neurotherepeutic strategies have been proposed including neurotherapeutic drug administration and/or cellular interventions. Among these experimental approaches, endogenously recruited or exogenously expanded NSCs have been proposed as next generation neuro-therapeutic targets for injury repair (figure 11). These cells represent a promising therapeutic adjuvant for regenerative therapy post injury, due to their ability to generate mature, functional neural cells able to replace degenerated ones [64], [65]. Several studies have focused on NSCs as a strategy to treat TBI. Transplanted cells into the site of injured brain show significant cells differentiation, migration, and long-term survival post transplantation with recognized improved motor and spatial learning post injury [66]. Therefore the long-term strategy may be required through direct transplantation of neural stem cells into the injured CNS that can provide a long-term survival and integration by mediating functional recovery through mechanisms such as bulk trophic support, cell–cell mediated repair and replacement of cells lost by injury [67].

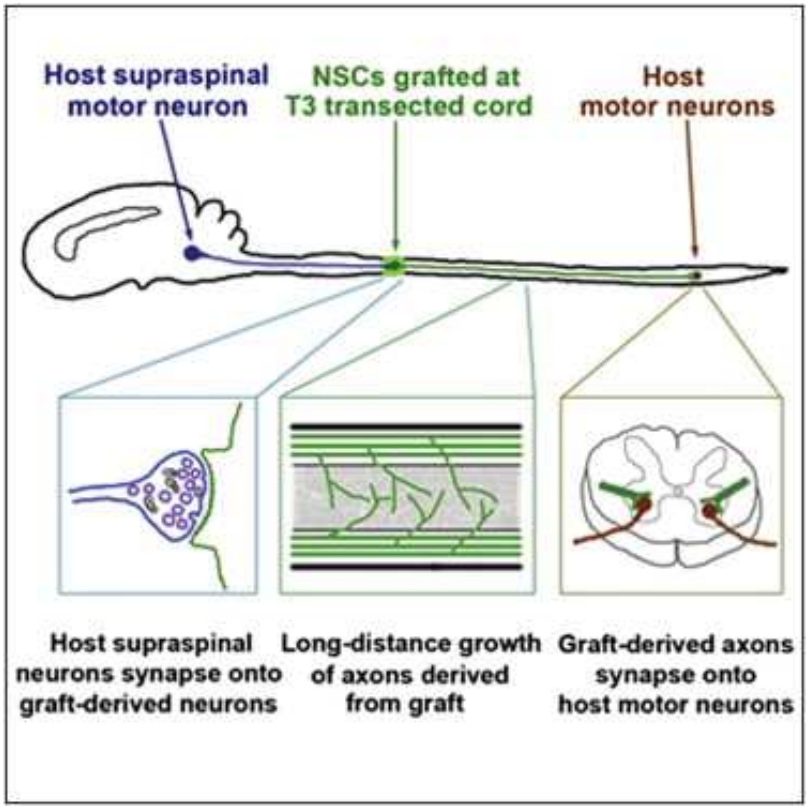


Figure 11: Stem cells transplantation as a strategy in treating CNS injury.
adapted from [67].

2.2 Stem cell niches out of the CNS

Beside the NSCs niche in the CNS, other stem cell niches reside in various organs including skin and intestine. These epithelial tissues undergo continuous cell replacement in a process called homeostasis [68]. Homeostasis is highly dependent from the stem cell niche that can regenerate damaged tissue following injury, facilitate rapid repair and prevent tissue over growth. Similar to NSCs, epithelial stem cells possesses the capacity for both long-term self-renewal and multi-lineage differentiation with a traditional paradigm of a unidirectional, hierarchal differentiation trajectory. Epithelial stem cells beginning with a multipotent self-renewing stem cell and proceeding through transit-amplifying cell stages and then into terminal differentiated state. The intestinal epithelium is a single layer of cells that extend invaginations (crypts) into the underlying connective tissues (figure 12A). Indeed, the intestinal epithelium is one of the fastest self-renewing tissues and completely regenerates within 3-5 days [69]. Intestinal stem cells (ISCs) are of two types,the LGR5+ rapidly cycling cells are located at the crypt base interspaced between Paneth cells [70], whereas slow-cycling, label-retaining HOPX+,LRIG+,BMI1+,TERT+, DLL1+ ISCs are located at the +4 position relative to the crypt base [70], [71]. These cells are migratory cells and can generate and differentiate into various cells types the absorptive or secretory lineages and finally into one of four differentiated cell types: enterocytes, mucin-secreting goblet cells, peptide hormonesecreting neuroendocrine cells and microbicide-secreting Paneth cells [72].

On the other hand the skin epithelium is a multilayered, stratified epidermis, and fully renewing every 7-10 days (Potten et al., 1987). The basal stratified layer is where epidermal stem cells (ESCs) reside (Fig.12B), and they initiate a transcriptional program of terminal differentiation while migrating upwards to generate to the spinous layer, the granular layer and, finally, the cornified layer of dead cells. In addition to the ESCs that maintain the skin epidermis, the hair follicle stem cells (HFSCs) maintains the skin hair follicles (Fig. 12B). The hair cycle starts with the quiescent HFSCs residing in the so-called bulge niche that are triggered to proliferate through complex signaling crosstalk with neighboring niche cells, and migrate to supply the cells needed for hair follicle down growth [73], [74].

The regulation of homeostasis and the response of stem cells to injury is coordinated by the crucial role of stem cell-to-daughter cell crosstalk. In another words, coordinated by the niche signals that originate from stem cell progeny. In the intestine the cross talk is between paneth cells that secrete important signaling molecules such as (WNT3, EGF and Notch ligand DLL4) and LGR5+ ISCs (figure 12A). Studies confirmed that LGR5+ ISCs can form differentiated

intestinal organoids if co-cultured with a paneth cell-enriched population or adding exogenous WNT3A [75]. Interestingly, WNT signaling alone is not sufficient to drive LGR5+ ISC self-renewal, but additional signals from R-spondins are also required. WNT is known to stabilize R-spondin receptor expression (LGR4, LGR5, LGR6), thus enabling R-spondin to promote stem cell expansion [76]. These findings suggest that stem cell progeny within the niche support stem cell functions through providing the most crucial signals such as WNT, this would facilitate robust proper stem cell activities. Similarly, the feedback mechanism – from progeny back to stem cells – also exists in the skin, where early HFSC progenitors signal back to HFSCs to drive their activity during hair regeneration. In this context, progenitor cell SHH sustains HFSC activation during the hair follicle growth phase (figure 12B) [77]. This provides a self-organizing feedback loop to control HFSC activation to the degree of hair follicle growth. Besides progeny stem cell-to- stem cell crosstalk, also stem cells themselves signal to their progeny or to themselves, in another word signals can originate from and to stem cells [78]. ESCs express several Wnt genes, and inhibition of WNT secretion leads to their premature differentiation, thus autocrine WNT signaling maintains the undifferentiated stem cell state during homeostasis [79]. Similarly, in the intestine, Notch signaling promotes LGR5+ stem cell proliferation, while preventing differentiation into the secretory cell lineage. In this context, deletion of Notch leads to secretory cell hyperplasia [80], while blocking WNT signaling in the intestine rescues this secretory cell hyperplasia [81]. So Notch signaling tunes local WNT activity, thereby coordinating balance between self-renewal and differentiation within the niche. Collectively, this shows the different sources of various factors and signals in intestinal and skin niches, and reveal how stem cell behavior is controlled to ensure precise lineage output responses to maintain or restore tissue integrity, characteristics that are shared with CNS niches.

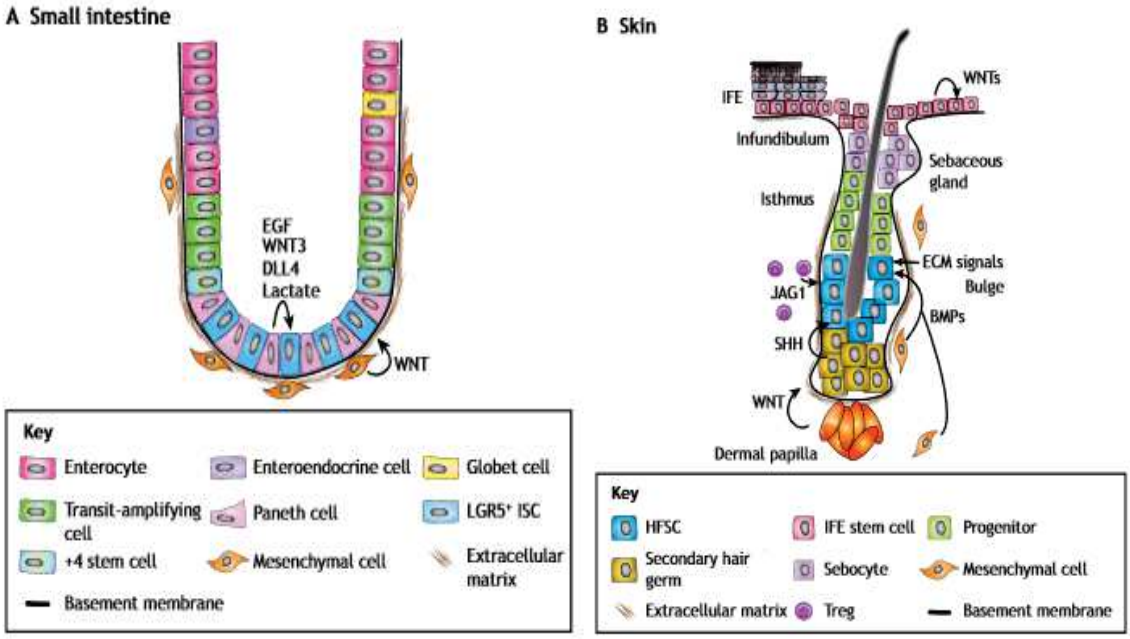


Figure 12: Intestinal and epidermal stem cell niches.
(A, B) Architecture of the cellular organization and the niche niche signals controlling lineage hierarchies and dynamics during homeostasis in intestine and skin tissues [73].

Chapter III: Neural Stem Cells in their Neurogenic Niches

3.1 Generalities

In the CNS, NSCs reside in a well conserved, organized, and specialized microenvironments called stem cell niches**.** The lifelong self-renewal of stem cells, and their differentiation into specific cell types are well preserved and supported by these highly controlled niches. Therefore, NSCs and their interaction with the environment participates in the constitution of the niche. Furthermore, Tissue remodeling requires a highly dynamic niches capable of changing location and characteristics overtime. Within the niche, intrinsic and extrinsic signals as well as cell–cell interactions are key regulators allowing feedback control of stem cell activation and differentiation. The accessibility of growth factors and other signals is regulated by the basal lamina or stromal cells where stem cells in the niche are anchored. Thus this anchoring play a key role in orienting cell division into one or both daughter cells depending on the plane of division, in which basal lamina form a substrate for oriented cell division [82]. In addition to this, there is an intimate association with endothelial cells, which regulate stem cell self-renewal and differentiation. Neurogenic niches are built so that NSCs can receive, integrate, and respond to signals from the surrounding microenvironment. Briefly, NSCs are (1) closely associated with the vasculature that makes neurogenesis associated with angiogenesis, (2) adjacent to a variety of neighboring cells, including their own neuronal progeny, resident mature astrocytes and microglia, and (3) in close contact with basal lamina components. Adult neurogenic niches maintain the stem cell pool, direct neuronal production, and provide protection of ongoing neurogenesis from possible external inhibitory influences [82]. Functionally, members of the vascular endothelial growth factor (VEGF) family can stimulate neurogenesis as well as angiogenesis. It has been unclear whether they act directly via VEGF receptors (VEGFRs) expressed by neural cells, or indirectly via the release of growth factors from angiogenic capillaries [83]. VEGFR3 is expressed by neural stem cells, neurons, glia, and ependymal cells residing in brain and spinal cord. For instance VEGF-C stimulates mitosis of VEGFR-3-expressing NSCs, and this ligand receptor coupling promotes subventricular neurogenesis, but not angiogenesis. Therefore, VEGF-C/VEGFR-3 signaling acts directly on NSCs and regulates adult neurogenesis, and may open potential approaches for treatment of neurodegenerative diseases [84], [85].

Eventhough, factors mediate these processes are still being elucidated, it is proved that both neural and non-neural cell types are key players [82].

3.2 Location of NSC niches in the adult brain

In the adult brain the primary regions of proliferation and active neurogenesis are sub-ventricular zone (SVZ) of the lateral ventricle and the sub-granular zone (SGZ) of the hippocampus. Therefore extensive work has been made to understand the reason behind these active two sites. Studies confirmed that those two regions are the NSC niches of the brain that contain a pool of stem and progenitor cells that produce the diverse cell populations of the brain (neurons and glia). In addition to that, recent work showed the existence of a third neurogenic niche that resides in the third ventricle by the hypothalamus.

3.2.1 Sub-ventricular zone niche

SVZ is composed of a thin layer of dividing cells that run along the entire length of the lateral walls of the lateral ventricles and is largely separated from the CSF by a layer of multi-ciliated ependymal cells. The SVZ niche (figure 13) is well characterized and extensively studied, where it is considered a persisting zone of active neurogenesis in the adulthood. Briefly, the neurogenic niche consists mainly of three cell types that differs ultra-structurally and in the activation state. Starting with B1 cells, the in-vivo postnatal and adult NSCs, are characterized by the expression of the astrocytic GFAP marker. Their radial glial morphology, apical process, and primary cilium put B1 cells in direct contact with CSF and blood vessels, representing two pools of extrinsic signals that regulate neurogenic activity. Also the apical end feet of B1 cells are surrounded by the ependymal cells a property that creates the hallmark « pinwheel » structure. In mid embryogenesis B1 cells are quiescent (qNSCs), a sub-population maintain its dormancy and another sub-population respond to an activation signal where they generate activated NSCs (aNSCs) or what is called transit-amplifying progenitor cells (TAPs or type C cells). Those cells divide several times before generating neuroblasts (mainly labeled with DCX) the immature neurons of the brain that migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB). These DCX+ cells will generate two types of inhibitory interneurons that they will integrate in the OB circuitry. This OB neurogenesis will form and maintain olfactory memory and odorant discrimination [86]. To a certain extant B1 cells are capable of generating oligodendrocyte lineage cells [87]. Other migratory pathways were also described in case of injury, several studies showed migrating neuroblasts from SVZ into the neighboring striatum and the site of injury [88]. The migration of neuroblasts through the RMS is highly dependent and regulated by the GFAP+ ciliated radial glial cells. In fact, B1 cells orient and guide the migration from the SVZ through a balance between chemo repulsants and

chemo attractants from the SVZ environment. For instance, neuroblasts are repulsed away from the SVZ to the OB by the SLIT proteins a chemo repulsive stimulation for axon growth and guidance [89]. In addition astrocytes and glial cells play a key role in regulating the dispersion and the speed of migration respectively. Neuroblasts are encapsulated by astrocytes and form a cordon like a glial tube to prevent their dispersion and their speed of migration is diminished by the migration-inducing activity (MIA) secreted by glial cells [90]. Furthermore, a coordinated pattern of interactions between extracellular matrix (ECM) cues, cell adhesion molecules, and cell-surface integrin signaling receptors regulate the maintenance and guidance of neuroblast migration. (1) Polysialated form of neuronal cell adhesion molecule (PSA-NCAM) responsible for maintaining the strength of the cordon like a glial tube. (2) Integrins and integrin ligand (tenascin-C) they convey the ECM-derived signals to the neuroblasts and fill the extracellular space between the migrating neurons and astrocytes in the RMS respectively [91].

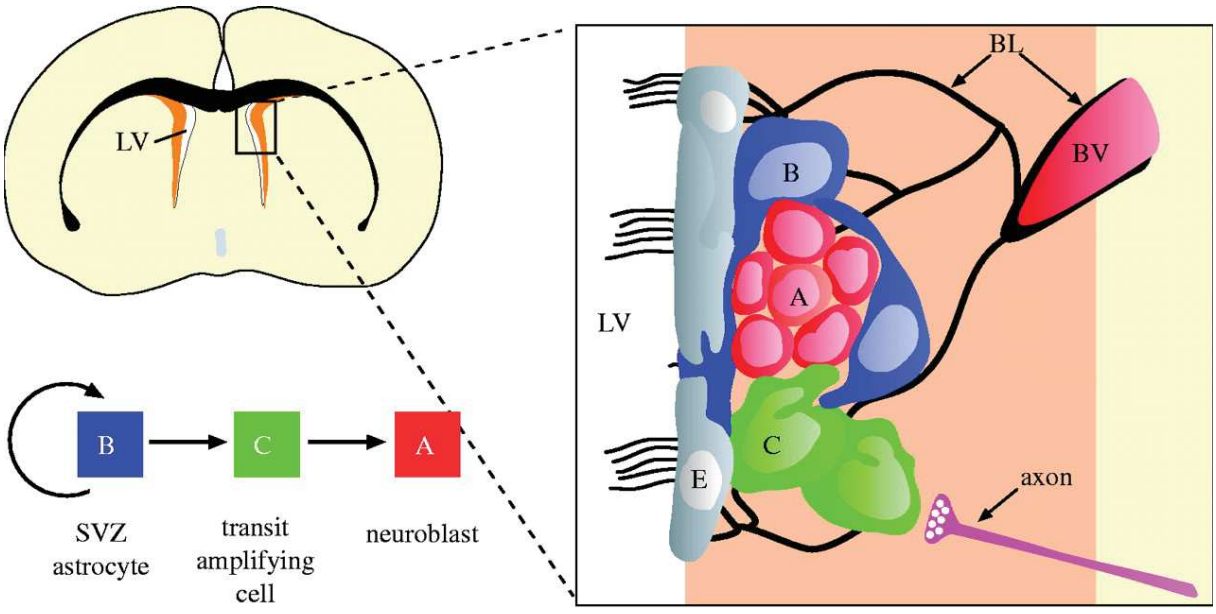


Figure 13: Anatomy and cell types within adult SVZ niche.
Schema represents a frontal section of the adult mouse brain showing the SVZ (orange), and the cellular types and their organization within the niche [91].

3.2.2 Sub-granular zone of the hippocampus

The dentate gyrus (DG) of the hippocampus is the second brain region where active neurogenesis occurs. Neurogenesis originates from the precursor population that resides in the SGZ niche (figure 14), found between the hilus and the granular cell layer that was described by Joseph Altman in 1975. The active proliferative spots are close to the vasculature suggesting

a role of blood vessels in regulating neurogenesis [92]. Adult hippocampal precursors within the niche are multipotent, they generate granule neurons, astrocytes, and oligodendrocytes in certain conditions. Briefly new neurons will migrate at a short distance to the granule layer where they extend dendrites to the molecular layer and axons to the mossy fiber path. There they are implicated in learning and memory [91]. Radial astrocytes or type1 cells are abundant in the SGZ with a rare dividing property. They express the astrocytic marker GFAP, and function as the stem cells of the SGZ. They send projections to the molecular layer and the contact blood vessels. They generate the high proliferating intermediate progenitor cells or type II progenitors, that give rise the mature functional neurons [93]. Type1 and type 2 cells differ morphologically and by the expression of markers. They share expression of some markers suggesting a reciprocal interplay between the two types. Unlike type 2, type 3 cells (neuroblast) are slowly and rarely proliferating and express neuronal markers where glial markers expression is lost [94].

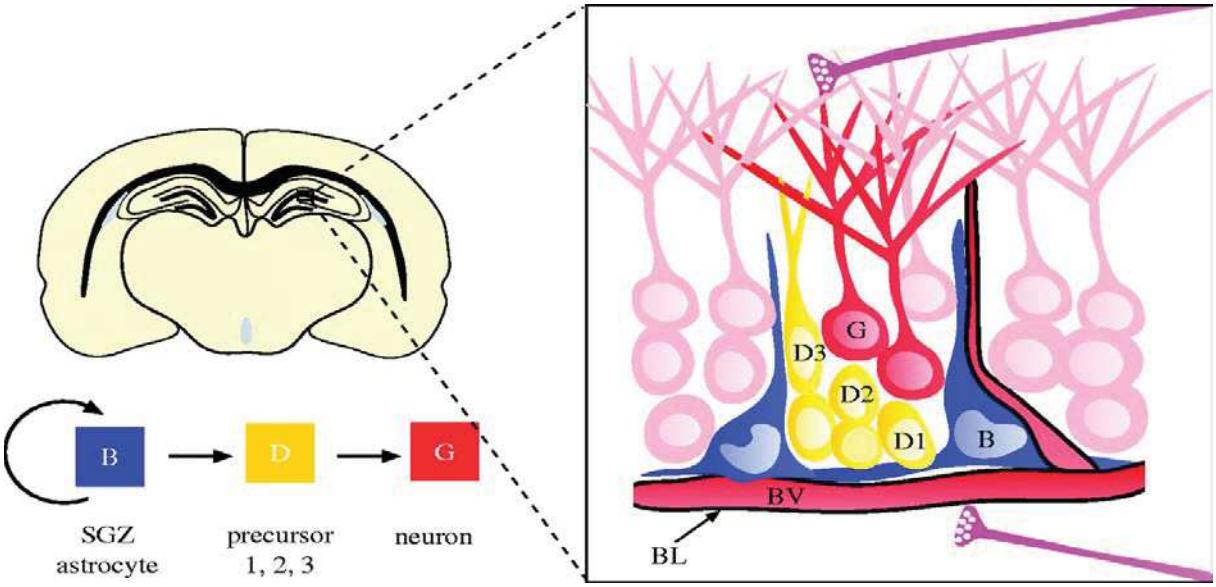


Figure 14: Anatomy and cell types within adult SGZ niche.
Schema represents a frontal section of the adult mouse brain showing the SGZ of the hippocampus, and the cellular types and their organization within the niche [91].

3.2.3 Third ventricle by the hypothalamus

Various reports showed that Adult brain harbors a third less studied neurogenic niche resides in the third ventricle by the hypothalamus (figure 15). Ependymal layer lining the third ventricles and harbors tanycytes, the stem cells of this niche. They are multipotent cells, under physiological conditions they generate neurons and glia, as well as neurospheres in-vitro [95]. Tanycytes are ciliated cells with elongated radial glial-like morphology with long basal

processes, resembling neural stem cells in other neurogenic niches. Such features created a similar stem cell niche-like cyto-architecture between SVZ and hypothalamus. However the regulation and neural stem/progenitor cell potential of hypothalamic tanycytes, as well as, the functional role of hypothalamic neurogenesis should be deeply investigated. In addition, the potential involvement of hypothalamic tanycytes in adult human neurogenesis needs to be addressed (M. Perez-Martin, 2010). But as hypothalamus is involved in sleep, circadian rhythm, and blood pressure, it is suggested that hypothalamic neurogenesis could be involved in the control of the body's energy balance [96]. Recent study showed that, hypothalamic neurons (pro-opiomelanocortin (POMC+)) innervates the V-SVZ niche. So enhancing the activity of those neurons will enhance the number of NKx2.1+ cells as POMC+ neurons directly contact NSCs as well as niche cells. As such, the effect on NSC proliferation caused by modulating hypothalamic POMC neuron activity could be due to direct stimulation of NSCs, volume transmission, or via niche cells. Hypothalamic neuronal innervation can target different spatial domains in the V-SVZ stem cell niche and selectively affect distinct pools of adult NSCs. Neural circuits from diverse brain regions may therefore underlie the regulation of heterogeneous adult NSCs by mosaically innervating the V-SVZ stem cell niche. Moreover, the hypothalamic neural circuits link physiological states to regional NSC proliferation and the production of discrete OB interneuron subtypes. As such, they provide a logic of how diverse physiological states may lead to on-demand adult neurogenesis [97].

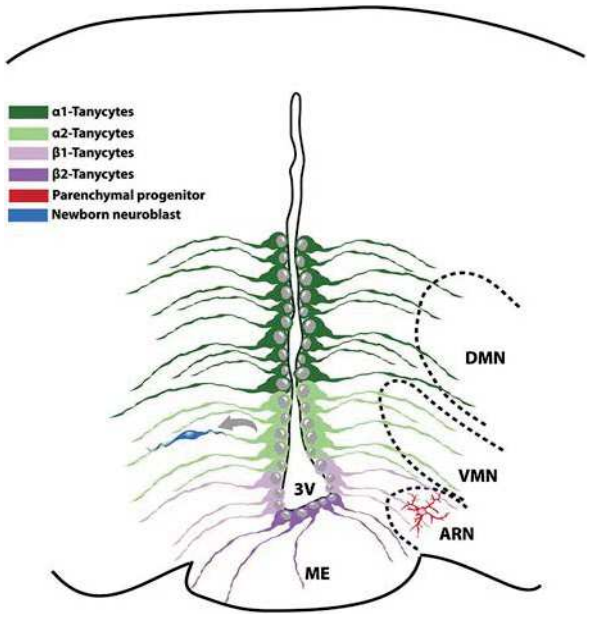


Figure 15: Anatomy and cell types within adult hypothalamic niche.
Schema represents a frontal section of the adult mouse brain showing the hypothalamus, and the cellular types and their organization within the niche [98].

3.3 Location of NSC niche in the spinal cord

Little was known about the location of the neurogenic niche of the spinal cord, as well as the regulation and function of this niche [99]. Stem/Progenitors Cells (SPCs) were residing in the central canal, a slow proliferating zone and the main source of NSCs (figure 16). The parenchyma of the spinal cord could be a niche where rapidly proliferating stem cells and glial progenitors reside [100].

The spinal cord has no defined sub-ependymal layer, in which ependymal cells are the main source of stem cells both in-vivo and in-vitro. Bruni and his colleagues identified various types of cells that constitute the ependymal cell niche [50]. Cells varies from cuboidal, to tanycytes and radial cells that contact the lumen, and to ciliated ependymal cells. Under physiological conditions the ependymal niche is quiescent, in which rare proliferation takes place, but this niche turns to be active in response to spinal cord injury, where the niche becomes highly proliferative and generate astrocytes and oligodendrocytes [50], [101]. In addition to that, cell transplantation studies, have demonstrated that, spinal cord derived NSCs are able to generate glial cells when grafted in the injured spinal cord, also they generated new neurons when grafted into the neurogenic hippocampus [102]. Thus at least a subpopulation of the ependymal cell layer possess NSCs properties with multipotent differentiation potential.

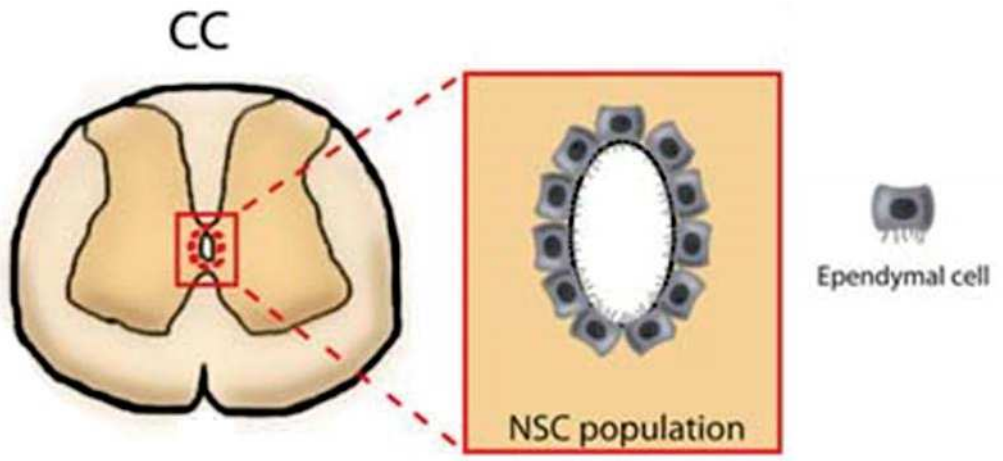


Figure 16: Anatomy and cell types within adult spinal cord niche.
Schema represents the adult mouse spinal cord showing the ependymal cells lining the central canal niche [103].

The ependymal neurogenic niche has been described in various species (rodents, macaque, and human), showing differences and similarities in the anatomy of the niche.

1- In rodents Alfaro-Cervello et al 2012 described the ependymal central canal by toluidine blue-stained spinal cord sections. The ependymal zone (EZ) is a pseudo-stratified epithelium

derived from the developing ventral neuroepithelium due to the fusion of the dorsal wall zones and is controlled by SHH signaling [104]. In the cervical part the central canal is rounded with a wide lumen, while the dorsal and lumbar central canal is collapsed with elongated shape, and the lumen is reduced [105]. Despite these anatomical differences, cell types and their organization is similar among the spinal cord parts. EZ cells are highly polarized with different morphologies and protein expression (figure 17). Similar to zebrafish, dorsal and ventral midline (tanycytes) cells have a radial morphology and characterized by a long filament process that extends to the pia matter [106]. While lateral ependymocytes have a cuboid or radial morphology with processes contacting the blood vessels. To summarize, ependymocytes within the EZ share common features: electron-dense cytoplasm rich in intermediate filaments, a nucleus with small chromatin clumps, and a radial expansion [105]. For detailed data see table 1.

	Ependymal	Astrocyte	Neuron
CONTOUR	Smooth, radial	Interdigitated, radial	Smooth, apical expansion
CYTOPLASM	Dark	Light	Medium
NUCLEI	Round-oval	Irregular	Round
CHROMATIN	Small clumps	Clumped	Lax
NUCLEOLI	3–4	1–2	1–2
RER	+	+	++++
GOLGI	+++	+	+++
MITOCHONDRIA	++++	++	+++
FREE RIBOSOMES	+	++	++++
INTER. FILAMENTS	++++	++	No
MICROTUBULES	No	No	+++
LIPID DROPLETS	Yes	No	No
CILIA	1–4	1	1
MICROVILLI	Yes	Yes	No

Table1: Morphological features of the various cell types in the adult mouse central canal.
+, few; ++, intermediate; +++, abundant; ++++, extremely abundant [105].

2- In non-human primates such as macaque monkey, the ependymal zone was described by Alfaro-Cervello 2013, using high-resolution electron and confocal microscopy. As in rodents, the primate central canal is surrounded by pseudo-stratified epithelium, with rounder and wider cervical canal, and a restricted elongated thoracic and lumbar canal. The EZ and peri-EZ consist of 5 cells types, in addition to astrocytes and neurons, there are three subtypes of ependymal

cells that differ in the number of cilia they possess (uniciliated, biciliated, and multiciliated). In contrast to the mouse EZ, non-proliferating multiciliated cells are the most abundant cell type in the monkey EZ, and reside in the lateral wall of the canal (figure 17). On the other hand, proliferating uni and biciliated cells reside in the dorsal and ventral roof of the canal. Similar to rodents, ependymal cells have long radial processes that contact the vessels, and characterized by the electron-dense cytoplasm. Despite the number of cilia, ependymal cells share the same cytoplasmic contents [105], [107].

3- In humans little data is available on the EZ, even though it was studied by von Lenhossék in 1891. Histological analysis on post mortem infant human sections showed a wide opened central canal [108], and a pseudostratified epithelium with two or three cell layers. Similar to rodents and macaque, roof and floor cells in the human possess a long radial processes that extend to the pial surface and express Nestin [109]. It's suggested that these cells could be involved in guiding descending and ascending axons, and could provide supply for neurons until vessels appear [110]. Like macaque, infants showed three subtypes of ependymal cells while adult humans showed only two subtypes of ependymal cells characterized by two or more cilia. Like macaque, multiciliated cells are the most abundant cell type in the EZ. Human appear to have astrocytes similar to those of mice central canal astrocytes, but central canal contacting neurons were not detected in humans [105], [107] (figure 17). Unlike rodents and macaque, no proliferation was observed in the human EZ using proliferation markers, or rare events of proliferation could be missed [107], [111]. The major difference between rodents and primates is the presence of hypocellular layer formed by the increased level of GFAP filaments around the EZ in primates [107], [112]. This layer also exists next to the SVZ niche thus considered a distinctive property of primates, and could be the reason behind the absence of regeneration in primates. Another interesting distinctive feature of the human central canal is that, the canal showed displaced ependymal cells resulted from the invagination and folding of the ventricular surface. Thus the central lumen is not patent and an obliteration process occurred at different levels of the spinal cord in humans, while it is maintained at the level of the medulla [113]–[115].

To conclude with the location and anatomy of NSC niche in the spinal cord, there is differences between ependymal central canal of rodents and primates. Of the major differences is the abundancy of multiciliated cells in primates, and this could be due to the larger lumen [116]. Another distinctive difference is the existence of hypocellular layer around the central canal that could be the main obstacle for regeneration in primates [114], [117]. However some

similarities between mice and rodents are observed such as the presence of proliferating uniciliated and biciliated cells to maintain length extension [105]. Finally, in this context it's necessary to go further in studying the EZ in these species and compare it to regenerative species such as zebrafish. So single cell sequencing will unravel the gene expression in the central canal to indicate the genes that are switched on and off, and to understand why humans have low or no regenerative capacities [118]. Thus a comparative approach of the EZ cells is a promising analysis to unravel the steps of inhibiting gliogenesis and switching regenerative neurogenesis after SCI and degeneration.

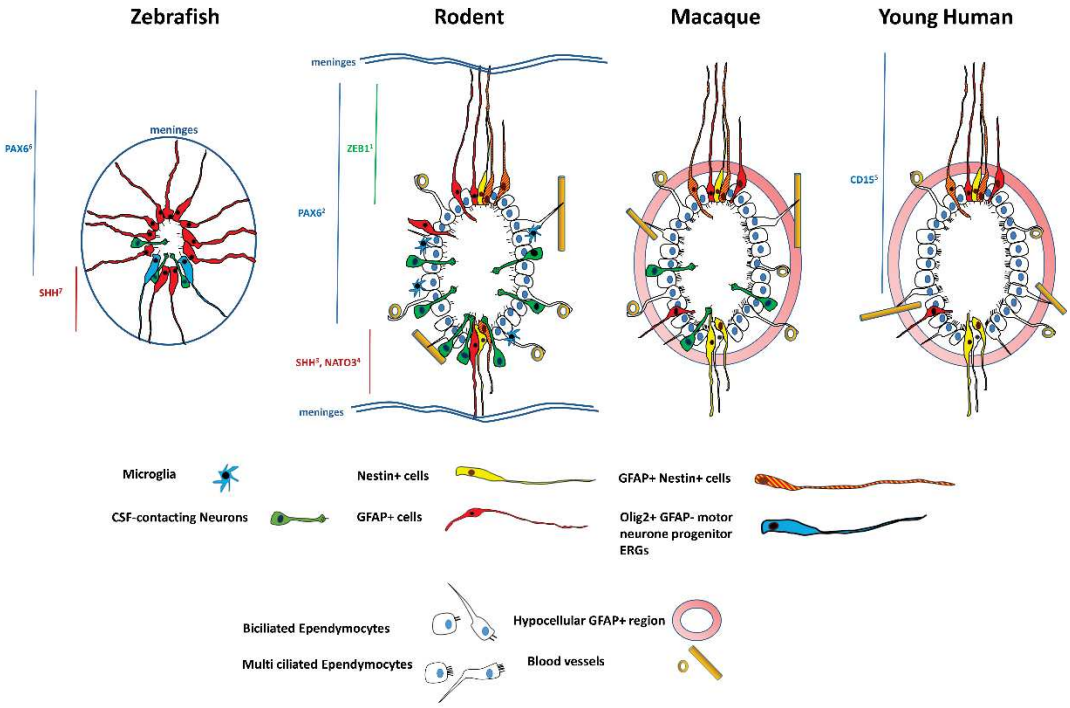


Figure 17: The organization of the ependymal zone across vertebrates.
A schematic representation showing cellular organization within the EZ of different species [117].

3.4 Heterogeneity of neural stem cells

NCS in their neurogenic niches are highly diverse and heterogeneous. Fiona Doetsch and colleagues studied extensively the heterogeneity of the brain niches, and showed the functional, regional and morphological heterogeneity within neural stem cell niches. Heterogeneity was studied using various transgenic models targeting stem cell markers in the niche, until the Single cell transcriptomic technology emerged that depends on sequencing hundreds to thousands of RNA in single cell [119]–[121]. This technology could also be coupled with the lineage tracing that provides a deep analysis of cell emerging, activation, and differentiation. However time is a key regulator of aNSC heterogeneity, where mRNA expression levels change over time, thus

affecting the single cell transcriptome analysis. Moreover the generation of various OB interneuron subtypes is highly dependent on the developmental periods (embryonic and adult). Adding to this changes in chromatin structure is time dependent, thus epigenetics is an additional factor of NSC heterogeneity that should be studied. So future studies are required to dissect NSCs heterogeneity over various factors to provide a better understanding of the normal brain function, plasticity and repair.

3.4.1 Heterogeneity in the sub ventricular zone

Heterogeneity in SVZ niche is studied extensively by Fiona Doetsch and colleagues, where they uncovered the NSC diversity, function and morphological differences [122], [123]. It's well known that SVZ postnatal and adult NSCs continue to generate neurons and OLs throughout life but the detailed mechanism of how NSCs differentiate is not well described, which is necessary to understand their behavior and function in vivo.

SVZ niche is composed mainly of quiescent neural stem cells (qNSCs), the term quiescent refers to the dormant state of the cells that maintain self-renewal, cell replacement, and DNA integrity. Thus they constitute the reservoir of various cell types that are recruited when needed. Over the last 15 years qNSCs was thought to be type B cells that express GLAST and GFAP markers, a structural and molecular feature of astrocytes. With their radial morphology GFAP+ cells possess a small process that extend a primary cilium to contact the CSF at the center of ependymal cell pinwheels (figure 18A), while from the basal side they send a long process that terminates at the blood vessels. Type B cells contacting the ventricles have a light cytoplasm and are quiescent, but when activated they give rise to the dividing transient amplifying cells (TACs) or type C cells that generate neuroblasts or type A cells. Neuroblasts will migrate to the OB and differentiate into OB interneurons. Adding to this scenario, type B cells also generate oligodendrocytes and astrocytes. The difference between the niche astrocytes and the other brain astrocytes was identified, to conclude that the niche astrocytes are themselves diverse and heterogeneous and have different structural and morphological features. So type B cells are now subdivided into quiescent type B1 and activated primed type B2, thus identifying additional markers is a key for better understanding astrocyte diversity and unmask new NSC in the niche [122], [124].

The detailed architecture of the niche indicates the various types of cells and the interplay between them. Using a GFAP-GFP mouse model in combination with antibody labeling against different proteins or performing single cell analysis on sorted cells was a good approach to

understand the interplay of cells inside the SVZ niche. Codega P et al., were able to identify and isolate qNSCs from their niche, through visualizing the SVZ astrocytes using the GFAP-GFP transgenic mice. As described before type B cells are in direct contact with the ventricle at the center of pinwheels, those cells are expressing GFAP that localized with the GFP, on the other hand they are immune-negative to S100β a marker of mature astrocytes. Those GFAP+ cells turned out to be CD133+, a marker of ependymal cells and cilia of type B cells, but the expression profile of CD133 was different among the GFAP+ cells. Interestingly a subpopulation of the GFAP+ cells expressed CD133 at the tip of the primary cilium, this population referred to the qNSCs. While the other GFAP+ population had a diffused CD133 expression along the apical surface and lacked a primary cilium, in addition they were immune-positive to EGFR and those represented the aNSCs. Both cell types had a radial morphology and were in direct contact with the ventricle and blood vessels. Therefore they identified two CD133+ astrocyte populations that had different protein profile, thus a different functional state (figure 18B) [122], [123], [125].

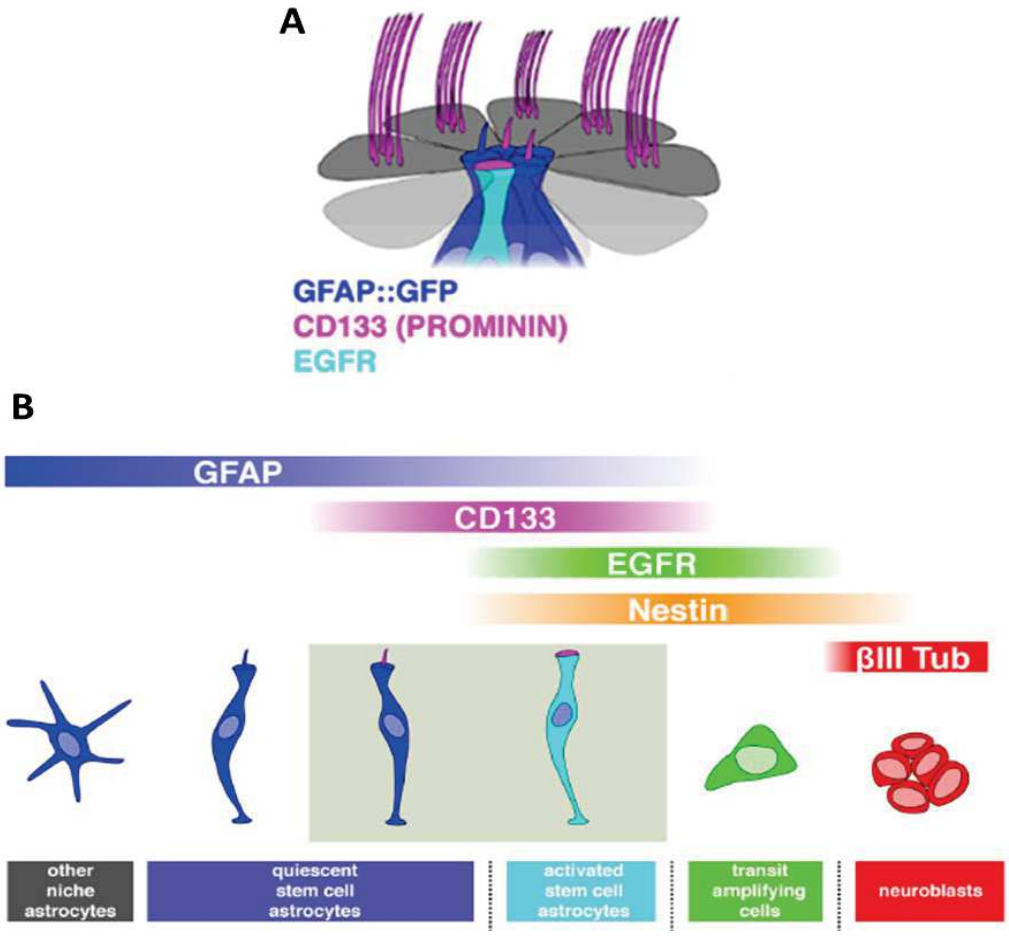


Figure 18: Properties of V-SVZ stem cells and their progeny in pinwheel structure.
(A) Schema showing different cell Types organized as pinwheel structure. **(B)** Schema showing the differentiation stages and properties of V-SVZ stem cells and their progeny [122].

These findings suggested that these two population of cells have different cell cycle properties. To confirm this hypothesis they immune-stained sorted cells with either proliferation markers or with a single pulse of bromodeoxyuridine (BrdU), both approaches confirmed that GFAP+ CD133+ lacked the expression of proliferation markers such as Ki67 and MCM2 and were BrdU- indicating that this population of cells is highly dormant. On the other hand, Ki67 and MCM2 were highly expressed during the G1 phase in the GFAP+ CD133+ EGFR+ population and were BrdU+, indicating the highly active dividing state of the cells.

Transplantation studies of qNSCs and aNSCs into the brain of adult mice showed that, both populations are able to generate neuroblasts that migrate to the OB through the RMS and were able to generate OB interneurons as well as were able to generate OLs. In fact aNSCs were able to engage in this process after only 1 week of transplantation while qNSCs took almost 1 month to generate neuroblasts. Therefore both populations are able to generate neurons and maintain the in vivo long term potential of the neurogenic niche, but they differ in their cell generation kinetics.

Combination of several approaches showed that qNSCs and aNSCs also differed in their molecular signature. Microarray analysis, qPCR, and immunostaining on purified cells showed that nestin, the intermediate filament protein and the hallmark of NSCs [126] is only expressed in aNSCs, where qNSCs had low levels of the nestin mRNA but were immune-negative for the nestin marker. But when qNSCs were plated in vitro, those cells up regulated the expression of nestin upon activation. Therefore upon activation, qNSCs gain the expression of EGFR and nestin and contribute to the lineage during regeneration. In addition to this, qNSCs were enriched in genes related to cell communication, response to stimulus, cell adhesion, and lipid metabolism, also they were enriched in factors known to be markers of quiescence such as VCAM1 and Lrig1. In contrast, aNSCs highly expressed cell cycle, transcription and translation, and DNA repair related genes. Moreover a subset of adult neurogenesis regulating genes were highly expressed in aNSCs and were a distinctive features of this population such as Dlx1, Dlx2, Sox4, Sox11, and Ascl1 [122], [123], [125].

In vitro both populations showed distinct behavior with reversible state capacity. aNSCs were characterized by the ability of colony formation unlike qNSCs that rarely form colonies and at low kinetics. Moreover qNSCs were rarely forming neurospheres and the neurosphere formation was delayed, on the contrary aNSCs significantly generated neurospheres. Both sources of neurospheres were multipotent generating neurons, astrocytes and oligodendrocytes, and able to survive several passages. Upon activation qNSCs are able to retain the property of highly forming neurospheres. In addition to that, in both cultures it was noticed that the culture

is heterogeneous, in other words neurospheres derived from qNSCs can give rise to GFAP+ CD133+ EGFR+ cells in addition to the GFAP+ CD133+ cells, similarly was noticed for neurospheres derived from the aNSCs. Therefore, the two populations can reversibly propagate between quiescent and activated state with the ability to generate all other populations, but differed in colony and neurosphere formation kinetics [122], [123], [125].

This study was followed by more detailed studies using high technologies to dissect the heterogeneity in the SVZ niche. Of these studies the one done by Ben Dulken in 2017 who used the single transcriptome analysis to define the dynamics in the adult stem cell lineage and showed how NSCs propagate along different states within the niche before generating mature differentiated cells. In this study they used the same transgenic mice (GFAP-GFP) and the same cell purification protocol described by codega et al, 2014, but instead they used another approach to identify heterogeneity depending on single cell analysis. So they identified and purified four populations of cells (figure 19):

Niche astrocytes are the GFAP-GFP+ CD133- EGFR- cells that were very similar to the GFAP-GFP+ CD133+ EGFR- qNSCs as describe previously [122]. aNSCs GFAP-GFP+ CD133+ EGFR+ were placed after qNSCs depending on the clustering and as cells propagate from quiescence to activation they upregulated Rpl3 a gene responsible for ribosomal genesis, then followed by the upregulation of cell cycle genes [127]. Interestingly, ki67 cell cycle markers showed that during transition from quiescent to activation there is an intermediary state characterized as the “cell cycle low” aNSCs as they expressed EGFR but not Ki67 unlike the “cell cycle high” aNSCs. So aNSC were subdivided in two different populations low and high cycling. As they improved the clustering of genes and referred to four-way stochastic gradient-boosting classification model [128], they were able to capture and understand activation, commitment, and dynamics of differentiation. So they identified a new subtype within the “cell cycle high” cells (GFAP+ EGFR+ KI67+), in which a subpopulation of cells expressed Dlx2 pro-neural marker responsible for neuronal differentiation [31], [82], [94]. Then aNSCs are followed by the last committed cells the NPCs GFAP-GFP- EGFR+ that are ordered at the end of cell hierarchy. NPCs are known to be the last state before the emergence of neuroblasts as they express markers of indicators of neurogenesis regulation such as Dcx, Sp8, and Sp9 and others. Therefore the new cell clustering showed that the cells propagate along a well-defined continuum starting from quiescence to activation then commitment and differentiation. Along this continuum cells are as follows: qNSC-like (Egfr−), aNSC-early (Egfr+Cdk1−), aNSC-mid (Egfr+ Cdk1+ Dlx2 low), aNSC-late (Egfr+ Cdk1+ Dlx2 high), and NPC-like (Dlx2+ Dcx+), ending up with five distinctive molecular states. Interestingly comparative single cell analysis

on in vitro cultured cells from different populations revealed that in vitro cells referred to the in vivo mid aNSCs with significant differences in the up regulation of inflammatory genes and cytokine signaling genes in the in vitro cultured cells [122], [123], [125].

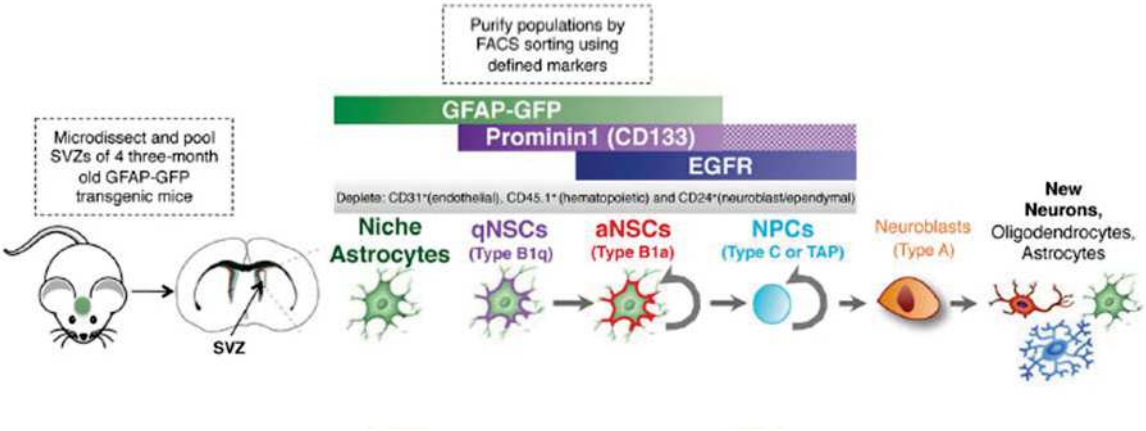


Figure 19: Cellular heterogeneity and linear progression in the SVZ of adult mice.
Scheme for the enrichment of astrocytes, qNSCs, aNSCs, and NPCs from the SVZs of adult mice [123].

3.4.2 Heterogeneity in the dentate gyrus

It is interesting to study hippocampal heterogeneity as it is highly related to better understand adult neurogenesis in the SGZ. Briefly kempermann et al 2015 showed that NSCs generate TACs that give rise to neuroblasts, then differentiate into mature neurons that migrate to the granule layer and integrate into cognitive functions such as learning and memory [129]. Various studies were done to unravel the heterogeneity of the hippocampal neurogenic niche using combination of NSC markers such as nestin, GFAP, and Prominin1 [123], [127], [130]. In a very recent study of Benedetta Artegiani, et al 2017 they used the nesting-GFP mouse model to FACS sort NSCs from the niche coupled with other NSC markers. They purified the GluR1-/cd24- cells a marker of mature granule cells and immature neurons respectively , and performed single cell sequencing [131]–[133]. In the hippocampal neurogenic niche several cell populations are described and subdivided into subpopulations (figure 20). First the neurogenic lineage is constituted by the NSCs and NPCs that express GFAP, Sox2, Sox9, and Id4; and DCX, Ccnd2, and Neurod1 respectively. Second oligodendrocyte lineage cells subdivided into OPCs (expressing NG2, PDGFRα, and olig2), and myelinating mature OLs (expressing Mbp, Plp, and Mog). Finally other glial cells subdivided into astrocytes and microglia represented by S100β and Csf1r respectively. In addition there are other populations identified such as interneurons (Reln), pericytes (Tbx18), and endothelial cells (Vwf). According to the clustering system they were not able to detect the intermediate states as the

cells progress from quiescence to activation, rather they were able to detect the change of marker expression along the continuum. At early stages cells were enriched in quiescent genes (Apoe, and Id3) and then along the continuum they gain the expression of activation genes (Fgfr3, and egfr) as described in the SVZ by Codega et al., 2014. Similar to the SVZ, proliferation markers were not expressed in early NSCs instead they were highly expressed in late NSCs as cells entered G1 and S phases (Codega et, 2014; shin et al, 2015). Also mature NSCs expressed low level of genes similar to NPCs such as Sox11 and Hmgn2 indicating that both NSCs and NPCs are distinct cell populations. NPCs are further subdivided into early (Ccnd2, and Hmgn2), intermediate, and late progenitors (DCX, and sox11), where early progenitors showed higher expression level of the proliferation genes (Ki67, and MCM2). These data unraveled the genetic and cellular signature of the hippocampal neurogenic niche [133], [134].

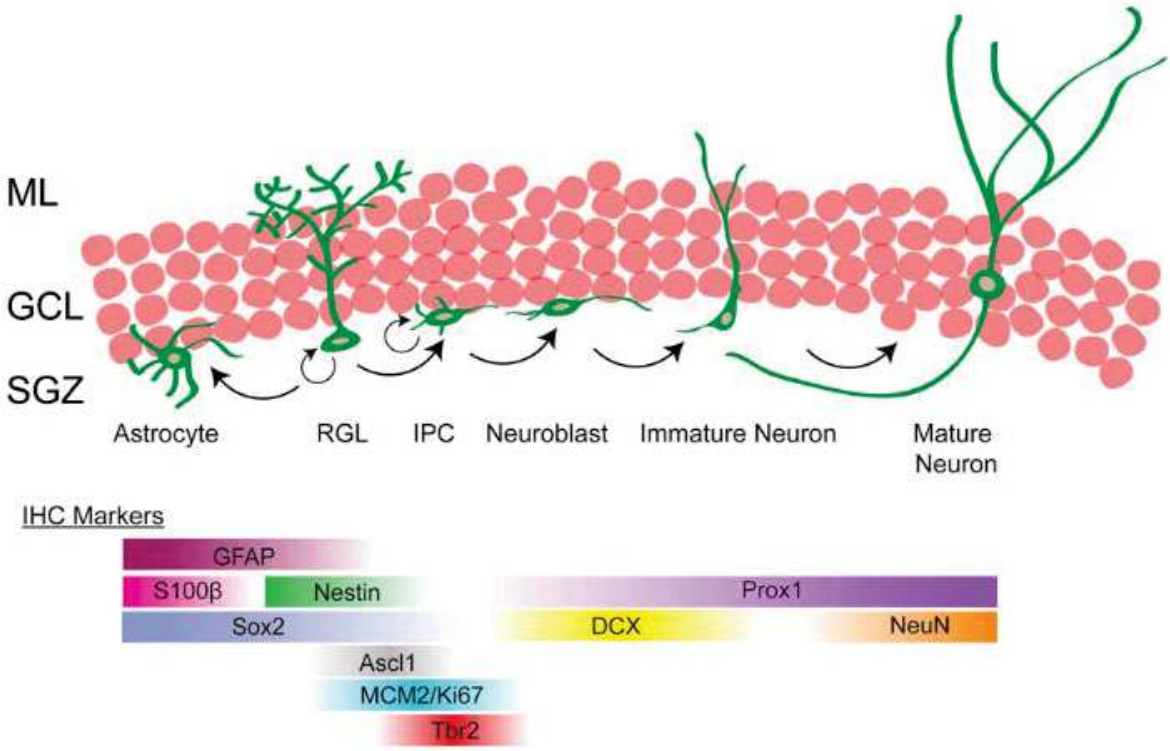


Figure 20: Cellular heterogeneity and linear progression in the SGZ of adult mice.
Scheme showing linear progression of radial glia-like cells (RGLs) in the SGZ, and the markers distinguishing different stages of the lineage progression in adult neurogenesis in the DG [134].

3.4.3 Heterogeneity within the ependymal

Epithelial cells lining the central canal wall in both postnatal and adult life and among various species remained poorly described. Several studies didn't provide a detailed description of central canal cells, for instance Mothe and Tator, 2005 identified ependymal and tanycyte cells

within the niche. While Meletis et al, 2008 identified three types of cells cuboidal, radial ependymal cells, and tanycytes. Another study done by Hamilton et al, 2009 who described new cell types in the canal niche apart from the ependymal cells and tanycytes, such cells are the astrocytes contacting the canal, oligodendrocytes, and the CSF contacting neurons. In his study he confirmed the expression of S100β and vimentin by both SVZ and canal ependymal cells, and few NG2+ and olig2+ cells were close to the ependymal canal cells.

Heterogeneity in the ependymal zone is species dependent, for instance it is less diverse in zebrafish compared to mice, and humans (figure 17). Unlike mammals, zebrafish shows an active regenerative neurogenesis, so it's interesting to show the cellular characterization of the EZ in zebrafish compared to the mammalian EZ. The zebrafish EZ constituted of one astroglia-like-cells called radial glial cells. Those ependymoglial cells are characterized by having their soma at the central canal a key feature of forming the ependymal layer, and with a long radial process extending vertically away from the canal (figure 17). Moreover those cells have 1 or 2 cilia where they express the foxj1 transcription factor responsible of cilia motility to maintain constant CSF flow. Combined together, these characteristics are at the origin of identifying the cells as ependymo-radial glia (ERG) [135], [136]. ERGs are enriched in GFAP, GLAST, and BLBP expression, in addition those cells are highly branched and their cilia sense signal from the central canal, thus they maintain a homeostatic function of ependymal cells and astrocytes [137], [138]. SCI studies showed that ERGs act as progenitor cells that generate several types of neurons unlike mammal cells that are destined to the glial fate [101]. Thus in response to injury ERGs generate several types of interneurons as well as motor neurons [139], [140]. Therefore ERGs are the promoters and only source of neuron generation and axon regeneration.

In rodents the central canal niche is highly diverse and consisted of several types of polarized ependymocytes (figure 17). Dorsal and ventral cells extended long processes and referred as tanycytes, and differed from the ependymocytes of the lateral walls that contact blood vessels and lack cilia [141]. Diversity among ependymocytes referred to distinct cellular morphologies and marker expression. Majority of Ependymocytes NESTIN−, VIMENTIN+, S100b+, CD24+, EphrinB1+, SOX2+, SOX9+ and CD133+ profile, while roof an floor radial midline cells showed low expression of vimentin and CD24 but high level of GFAP expression. Additional marker analysis revealed a dorsal-ventral and rostro-caudal regionalization, for instance, NKx6.6, Nkx2.2, and NATO3 are mainly expressed ventrally in the niche, while PAX6 covers the dorsal and the lateral walls of the canal only [105], [142]. This regionalization is maintained from development till adult stage [143]. In addition to the identified ependymocytes, the EZ niche harbors two non-ependymocyte cell types. First there are the

central canal contacting astrocytes (Accs), they are colonized in the dorsal and ventral canal and unlike ependymocytes they possess cilium. They extend long processes and they are heterogeneous population of cells, where some Accs express vimentin and nestin intermediate filaments [105], [144]. Second there are the CSF contacting neurons (CSF-N) that reside at different levels of the spinal cord. This cellular type is heterogeneous as it has different location, origin, function, and properties, and they are of two types lateral and ventral CSF-Ns [145], [146]. They are not a result of neurogenesis rather they are generated from two ventral domains of the spinal cord lately during development [146]. In addition to the immature neuron marker DCX, CSF-N maintain the expression of several transcription factors such as such as NKX6.1, NKX2.2, FOXA2, GATA2/3 that probably maintain the immature electrophysiological properties of these neurons [145], [146]. These neurons express functional ATP-gated P2X2 receptors, as well as high levels of the polycystic kidney disease-like channels (PKD2L1 and PKD1L2), which are distinctive markers for these neurons [147]. It has been confirmed that CSF-N have a GABergic property with an extended axon to the caudal part of the spinal cord and a small process extended to the lumen terminated by a large vesicle-containing budge. The expression of ion channel markers and their anatomical structure could explain the role of these cells. Due to the channels, these cells could respond to pH, osmolarity, and mechanical stimulation. While the existence of the budge inside the lumen is an indication of the secretory role into the CSF, thus they could be involved in the CSF composition and flow movement. Finally it was reported that in other CNS niches GABAergic signaling regulates quiescence and proliferation, so it has been suggested that, those CSF-N could have a similar role in the ependymal niche [148], [149].

In human and non-human primates (macaque), cellular heterogeneity has been described using electron microscopy, MRI, and marker analysis. Where most of the studies done on children and young individuals, data showed nearly a patent and a similar canal niche to that described for macaque and rodent niches with some particular properties (figure 17) [107], [143]. However, few studies have been reported that, the adult human canal niche has a completely different cellular structure and pattern. In adults the patent canal niche is almost absent, rather they have a randomly distributed morphologically heterogeneous cells (astrocytes, ependymocytes, and rosette pseudo-canal cells) [107], [111].

Although the pattern of the spinal cord niche is different among children and adults, it is highly heterogeneous at both ages. Three major characteristics define the niche of humans related to the cellular heterogeneity and organization (figure 17). Starting first with the active gliosis due to the presence of astrocytes represented by the GFAP+ cells that form a dense mesh around

the ependymal accumulation with dorsal, ventral and lateral extensions. The dense mesh formed by the GFAP+ cells is known as hypocellular layer, and could be responsible of inactive neurogenesis.

A second feature is the presence of two subtypes of vimentin+ ependymocytes (two cilia or multi-ciliated cells). These are protoplasmic cells with eccentric nuclei expressing CD15 and GLAST and found in masses with beta-catenin+ junctions. Finally, the third feature is the presence of pseudo-canals or rosettes that are oriented around a highly vascularized mass of cells (presumed lumen) and separated from the hypocellular layer. Only a subset of these cells are vimentin+ indication of heterogeneity within this population. Of note the proliferation within the human niche was absent as no Ki67+ cells were observed unlike rodents and macaque, but bad tissue preparation could mask low proliferating cells [107], [111], [150].

According to several marker studies, the human niche is highly diverse with a highly conserved architecture. Like rodents and zebra fish only a subset of EZ cells express nestin or GFAP, also some markers are expressed only dorsally (CD15, and PAX6) others are expressed dorsally and ventrally (NESTIN), thus these data showed cellular heterogeneity and dorsal-ventral regionalization within the human EZ niche. Of the other markers expressed in the EZ niche, are those typically expressed by immature NSCs such as vimentin, FOXA2, SOX2 and SOX11 [104], [142], [151].

Other studies confirmed the existence and persistence of neural progenitor cells in the adult human spinal cord. Dormard et al., generated nestin+/sox2+ neurospheres in vitro, these neurospheres were multipotent and able to generate astrocytes, oligodendrocytes, and neurons. As they were unable to be passaged those cells were derived from proliferation-limited progenitor cells. While Mothe et al., were able to generate same multipotent neurospheres that could be passaged several rounds using different culture conditions [56], [112].

To conclude with this part, several studies showed the structural and anatomical, cellular composition, and genetic profile differences in the EZ niche among the three species. The fundamental differences are likely existing between progenitor cells among species. So the cellular heterogeneity and organization is highly conserved and different, this created what is called the regenerative and non-regenerative species.

3.4.4 Functional significance of spinal cord ependymal cells

The EZ niche has been described and reported as a highly heterogeneous pool of cells. In the SVZ stem cells are self-renewing by symmetric division from either unipotent or multipotent progenitor cells. Niche environment, physiological cycles, and pathological conditions regulate the state of stem cells, starting from quiescence to activation and then differentiation in case of active neurogenesis [122], [152]. In the EZ of spinal cord the scenario is more complex, ependymal cells are latent neural stem cells with well characterized stem cell properties [50]. So one question to assess is whether all ependymal cells are stem cells or it's only a subpopulation of ependymal cells?

To answer this question, Alfaro-Cervello et al showed by electron microscopy that, unlike SVZ ependymocytes, the spinal cord ependymocytes self-duplicate with a degree of differentiation. On the other hand, in vitro studies by Weiss et al provided evidence about the existence of a subpopulation of NSCs in the EZ through the neursophere assay [105]. The generation of EZ derived neurospheres were confirmed by several groups and using various strategies.

The first strategy was the microdissection of the EZ central canal region, in which they confirmed the ability of cells to generate neurospheres that can be passaged for a long time (figure 21) [153]. The second strategy was the use of cell surface markers (CD24, and CD133) to pool out specific types of cells, interestingly these strategy was able to give an idea about cell types that were able to generate neurospheres in culture [154].

The third strategy is the use of transgenic animals that label a specific cell type in the EZ, where it was a good strategy to follow several NSCs in the EZ and whether they were able to generate neurospheres or not. The hGFAP-GFP mouse or the hGFAP-CreERT2 are widely used, and they showed that GFAP-GFP+ cells were able to form neurospheres and generate astrocytes and neurons in culture (figure 21). But one difference is that whether they have limited or unlimited self-renewal capacity, and this discrepancy could be due to the use of different design of the transgene or due to the different culture conditions [144], [153]. Where fiorelli et al suggested the existence of another source of neurosphere-forming cells, yet to be confirmed. But this suggestion was confirmed recently by Xu et al where he showed the formation of neurospheres by Oct4+ primitive NSCs in the EZ [155]. Another transgenic mice models were used to monitor other ependymocyes, foxj1 and nestin were good candidates. Nestin-CreERT and Foxj1-CreERT mice [50], [156], both transgenic mice showed that, foxj1+ and Nestin+ cells were able to generate multipotent neurospheres. Interestingly combining several data from various study they showed that, Foxj1 is expressed in most of the EZ cells including subset of

the GFAP+ cells [157]. This confirmed the hypothesis of the generation of neurospheres of various cell origins. Thus it is interesting to assess whether Foxj1+/GFAP+ and Foxj1+/GFAP- cells have different neurosphere-forming ability. Another group they used the recombined Glast-CreER mouse, Glast labels type A pericytes and a small subpopulation of ependymal cells [105], [158]. Glast-CreER cells were residing in the dorsal EZ where proliferating ependymocytes are mostly concentrated. So BrdU injection of this mouse model confirmed the proliferating state of GLAST cells, but the rate was different from other ependymocytes, suggesting the existence of other proliferating subpopulation of ependymocytes. Moreover those GLAST cells were able to generate neurospheres and could be maintained over four passages, thus suggesting their limited self-renewal capacity. Unlike the GFAP+ neurospheres that could propagate for a long-term [153], the GLAST+ cells were better considered as neural progenitor cells [144]. The same group represented by Sabelström H, et al they used the Troy-CreER mouse, troy is a tumor necrosis factor receptor that labels a small subpopulation of ependymocytes and subset of pericytes. Troy+ cells were quiescent in vivo, but unlike GLAST cells they generated neursopheres that are maintained over several passages, confirming their sustained self-renewal potential.

In conclusion, spinal cord ependymal cells have different ability of forming neurospheres with different differentiation abilities, thus ependymal cells are functionally heterogeneous showing proliferating progenitors and quiescent latent stem cells.

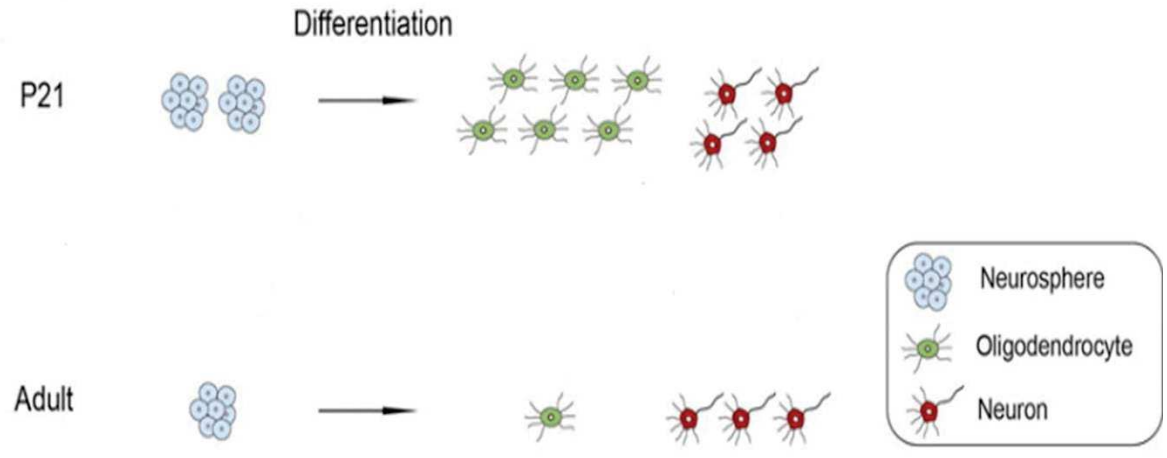


Figure 21: Schematic diagram of neurospheres derived from spinal cord cells in juvenile and adult mice.
Juvenile ependymal cells have higher intrinsic self-renewal capacity compared to adults in vitro, and can generate oligodendrocytes and neurons after differentiation [156] .

Chapter IV: Molecular regulation of the NSC niche in the spinal cord

Neural stem cells have the ability to self-renew and differentiate along multiple lineages, driving tissue homeostasis and regeneration. These processes are highly controlled by extracellular signaling molecules and the local NSC microenvironment, the "niche." There is a wide spectrum of niche-derived biochemical, and mechanical inputs that define stem cell states during morphogenesis, homeostasis and regeneration, and highlight how these diverse inputs influence stem cell plasticity [73].

Although neural stem cells have the highest potential to generate distinct progeny, they are themselves slowly cycling (quiescent) in adulthood, and through this behavior they regulate the maintenance of tissue homeostasis and regeneration throughout life. In the adult SVZ niche, qNSCs are found in the ventricular wall while activated NSCs (aNSCs) are found in the periventricular region, suggesting that unique cues in those microenvironments tightly regulate the positional identity of quiescent and activated NSCs (figure 13 and 19) [122], [159].

While several molecules produced by the niche cells have been identified to regulate adult neurogenesis, a systematic profiling of autocrine/paracrine signaling molecules in the neurogenic regions involved in maintenance, self-renewal, proliferation, and differentiation of NSCs has not been identified. These studies further suggest that certain signaling cues in the NSC niche govern the neurogenic potential of NSCs. However, the nature of niche signals is not fully elucidated.

In the spinal cord, multiple signaling pathways regulate the development and early patterning processes. These pathways involved in regulating cell division, rearrangement, and differentiation that are coordinated in time and space. Of these pathways, three essential signals are identified and play a crucial role in the development and and are retained in the adult stage; the Wnt/B catenin, the Bone morphogenetic protein, and the Sonic hedgehog pathways (figure 22A) [160]–[162]. These pathways are known to shape the EZ during the developmental process and maintained in the adult stage, in which they regulate the NSCs in their niche.

4.1 Integration of Wnt/BMP signaling

Wnt/BMP signal integration regulates various processes in the spinal cord during development and patterning. The early patterning in the dorsal spinal cord requires BMP gradient. As BMPs are expressed in the roof plate (figure 22A), the dorsal-most portion of the neural tube, dorsal progenitor cells are specified by BMP signals. These signals are known to drive mitogenic Wnt

gradient from the dorsal midline of the neural tube. The BMP/Wnt signals then influence domains of Wnt ligand, receptor, and antagonist expression that regulate cell cycle influencing progenitor expansion [163], [164]. As Wnt signal drive cells to exit the cell cycle, cells migrate and adopt neural identities, where the dorsal identities involve various transcription factors at the early stages (Pax7) and late stages (Ngn1 and Mash1) [165]. So patterning is regulated by BMP, linking this process to neuronal differentiation via Wnt-mediated proliferation (figure 22B). In addition to this, the β-catenin component of the wnt pathway plays an essential role in proliferation, cell-intrinsic and environmental properties might change over time during development, thus modulating the response of the cells [166]. The continuous growth of the dorsal spinal cord is predicted by the presence of Wnt gradient along the dorsoventral axis and the closure of neural tube closure and spinal cord formation has to be terminated in the dorsal-most spinal cord through limited neuroepithelial expansion. So these processes are controlled by the rate limiting steps in the previously mentioned signaling cascades [167]. Ille et al showed in his study that Wnt-mediated proliferation can be antagonized by BMP signaling. Moreover, BMP-dependent differentiation is encountered by Wnt, indicating that proliferation and differentiation in the dorsal spinal cord are controlled by cross-inhibitory interactions between Wnt and BMP signaling (figure 22B). So the integration of Wnt/BMP signal regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord [168].

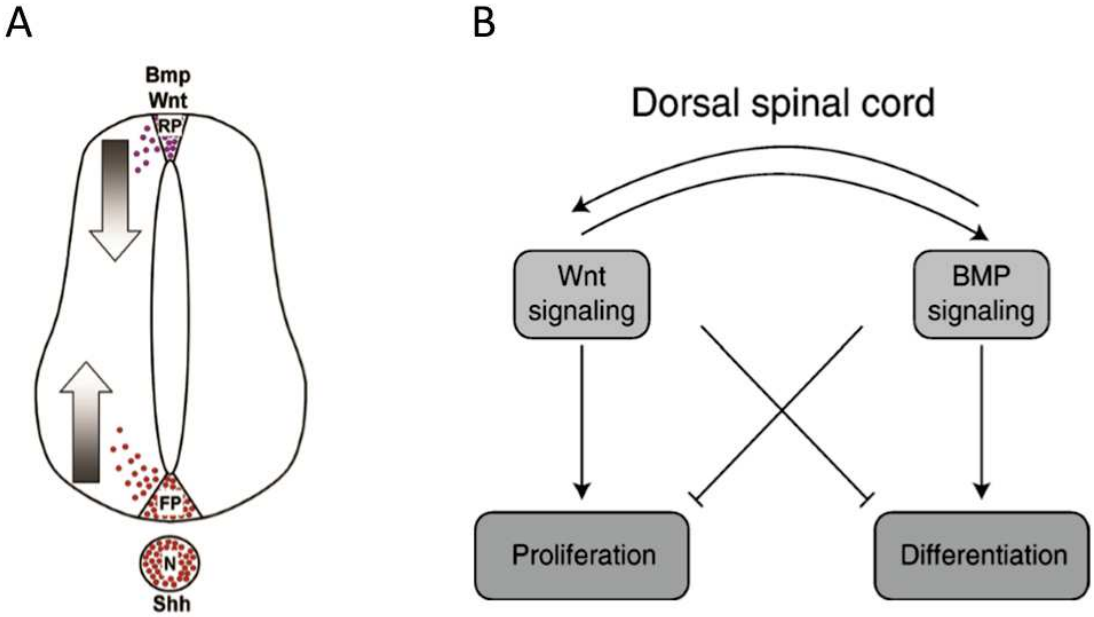


Figure 22: Signal cross-talk in the spinal cord.
(A) Dorsal Wnt/BMP VS ventral Shh. **(B)** Cross inhibition of proliferation-inducing Wnt signaling and differentiation inducing BMP signaling [168], [169].

Another process that requires Wnt/BMP signal during development and patterning is the specification of spinal cord neurons [170]. A combination of various bHLH transcription factors like Olig3, Mash1, and Ngn1/2 and homeobox factors like Gsh1/2 reveal distinct progenitor domains (figure 23A) [171], [172]. Six types of interneurons, dI1–dI6, are identified in these progenitor domains (p1–p6). These neurons are located in the mantle zone laterally and can be distinguished by the expression of homeobox transcription factors such as Foxd3, Isl1/2 and Lbx1 (figure 23A) [165], [172]. For instance, Olig3 transcription factor is expressed in p1–p3 progenitor cells and it is essential for correct and normal development of the dorsal interneurons. A mutation of *Olig3* in mice decreases the number of dI1 neurons and abolishes the generation of dI2 and dI3 neurons [172]. On the other hand, Mutation of *Math1*, a transcription factor expressed in p1, abolishes the specification of dI1 neurons [173], [174], while double mutations of *Ngn1/Ngn2* affects the generation of dI2 neurons [174]. Various studies and lines of evidence showed that members of the BMP family, like Gdf7, pattern the progenitor domains in the dorsal spinal cord and specify dI1–dI3 neurons [175], [176]. While, Wnt1 and Wnt3a members of the Wnt family, were also implicated in the specification of dorsal neurons, where double mutation of *Wnt1/Wnt3a* caused a reduction in the generation of dI1 and dI3 neurons is observed [177]. In addition to this effect Wnt signals in spinal cord exert a strong proliferative activity on progenitors secreted it, however, it is difficult to assess to what extent Wnt signals are essential for patterning or proliferation of dorsal progenitors [178]. Zechner et al showed in his study that the expression of *Olig3* is controlled by canonical Wnt signals in the dorsal spinal cord, where *β-catenin* gain-of-function induced a massive expansion of the *Olig3* expression domain and the appearance of extra dI2 and dI3 neurons. This implies that *Olig3* is required for the β-catenin-induced generation of supernumerary dI2 and dI3 neurons, but not for the enhanced growth of dorsal progenitors. Again the integration of Wnt/BMP signals coordinately control the expression of the transcription factor Olig3 and the specification of dorsal spinal cord neurons [170].

Moreover, little is known about the cellular origin and the molecular signals that regulate spinal cord ependymal cells. Various labs focused on this point including Xing et al who demonstrated and characterized the Wnt-responsive progenitor cells throughout spinal cord development. Also he showed that this population of cells is restricted to the dorsal midline and give rise to dorsal ependymal cells in a spatially restricted pattern. Genetic loss of function of β-catenin and inhibition of Wnt secretion in Wnt-activated ependymal cells resulted in impaired proliferation. So at the postnatal and adult stages, ependymal cells continue to exhibit Wnt/β-catenin signaling activity that promotes ependymal cell proliferation. Wnt/β-catenin signaling has been

also implicated in determining dorsalization of the postnatal subventricular zone and neural stem cell specification into oligodendrocytes and glutamatergic neurons [179]. In the report of Xing et al and by using genetic lineage tracing, they revealed the developmental origin of ependymal cells in the spinal cord. In the contrary to previous reports they pointed that ependymal cells are not derived exclusively of ventral origin of ependymal cells [180], rather they may retain positional identities in relation to their neural progenitors. In fact, the vast majority of ependymal cells in the postnatal and adult spinal cord express the Wnt/β-catenin signaling target gene *Axin2*, as well as Wnt ligands. The continued expression of Wnt/β-catenin signaling is important for both ependymal cell formation and regulation, where Wnt/β-catenin signaling regulates ependymal cell development and adult homeostasis [181].

In vertebrates, another transcription factors could be implicated in the patterns of neuronal differentiation set during the development of the CNS that form the basis of functional neural circuits in adults. For instance, PAX6 and MSX1 transcription factors (TFs) are among the first to be expressed in the dorsal proliferating neuronal progenitors [182]. Followed by the basic helix-loop-helix (bHLH) pro-neural TFs that are expressed within stripes and organized along the dorsoventral axis of the neural tube. There, they act as specifiers by exerting a neuronal identity on the cells, aligning their future location in the spine, and regulating their connections to their specific target [183]. Thus, the three dorsal most progenitor pools, known as dp1, dp2 and dp3, are respectively specified by Atoh1 , Neurog1 and Neurog2, and Ascl1 [174]. The sharp expression profiles of these four genes is driven by the morphogenic gradients of BMP and Wnt proteins secreted from the RP as well as the repressive interactions between the bHLH TFs [184]. Whereas the expression of Msx genes in the early stage of the neural plate, then during neuronal patterning and neurogenesis in the dorsal neural tube, the function of TFs *Msx1*, *Msx2* and *Msx3* remains unclear. Later after development, *Msx1* and *Msx2* expression domains become progressively restricted to the RP, whereas *Msx3* is expressed in the dorsal part of the spinal cord excluding the RP [185]. Later on various studies described the function of Msx genes by analyzing spinal cell fates within mouse embryos mutant for *Msx1* and *Msx2*. They showed that these two TFs interfere essentially during dorsal spinal cord neurogenesis, and they are necessary for *Atoh1* expression and dI1 interneuron generation. In addition, the expression domains of a set of TFs (*Pax7*, *Olig3*, *Neurog1* and *Ascl1*) is mispatterened in the absence of Msx1 and Msx2. Whether BMP signal is maintained and the function and the expression of MSX is reserved in the adult spinal cord, is still an open question to be answered. All together, these studies showed a novel function of *Msx1* and *Msx2* as transcriptional activators acting

upstream of Atoh1, and provided new insights into the transcriptional machinery control of spinal cord patterning by BMP signaling [186].

4.2 Integration of SHH signaling

Understanding the mechanism of how dorsal spinal cord ependymal cells are patterned and specified, led the way to uncover and understand the mechanism that control the neuronal identities and how positioned in a defined manner within the ventral spinal cord. The mature characteristic features of neurons in the adult organisms are coordinately regulated and gained during early development [169], [187]. A set of genes are identified and known to regulate the expression of these features, for that the generation and specification of ventral neurons during development is a precise and reproducible multistep process that occur in a spatiotemporal manner [188], [189]. The proper functioning of these processes is mediated by the main extrinsic activity of the Shh protein secreted from the floor plate (figure 22A). In the ventral spinal cord, Shh, patterns the generation of motor neurons and certain classes of ventral interneurons in a concentration-dependent manner [189]. Neurons are generated progressively more in ventral regions of the neural tube and required correspondingly higher concentrations of Shh for their induction. However, in Shh deficient mice, motor neurons and ventral interneurons fail to develop [190]. In the ventral part, a set of homeodomain TFs expressed by neuronal progenitors respond and interpret Shh signaling. Indeed, subdivides the ventral spinal cord into five progenitor domains, each of which generates a distinct class of post-mitotic neurons (figure 23B) [191]. Based on their mode of regulation by Shh, these homeodomain TFs can be categorized into class I and class II factors. Shh signaling represses the class I proteins (Pax7, Dbx1, Dbx2, Irx3, and Pax6), whereas the class II proteins (Nkx6.2, Nkx6.1, Olig2, Nkx2.2, and Nkx2.9) are enhanced by Shh signaling [192], [193].

Both in vivo and in vitro, expression of Shh can induce the differentiation of motor neurons and ventral interneurons, while eliminating Shh signaling blocks their differentiation. Although Shh can induce all ventral cell types, the generation of the dorsal-most interneurons of the ventral neural tube is Shh signaling independent. Instead retinoids from the paraxial mesoderm or from neural plate cells can induce these interneuron subtypes. So retinoid signaling seems to play a sequential role in spinal cord development, initially imposing the identity of spinal cord and later specifying the identity of some of its component neurons [187].

Shh signaling acts through the activation of Shh effectors namely, Gli transcription factors, that helps create a pattern of neural progenitor domains along the dorsal-ventral axis [194], [195].

There are three Gli proteins, identified as the principal transcriptional effectors of the Hedgehog pathway, where Gli1 and Gli2 act as transcriptional activators, whereas Gli3 functions primarily as a repressor [104]. However, Gli2 can function as a repressor in the absence of Gli3, and conversely, Gli3 can act as an activator in the absence of Gli2. Thus, the ratio of activator and repressor forms of Gli proteins can control and regulate the effect of Shh signaling within the embryonic spinal cord.

Shh gradient is sensed by a specialized cell structures called primary cilium, that mediates Gli processing and activity, which in turn activates target gene expression. These ciliary structures are important in Shh signal transduction as their loss leads to defects similar to those caused by mutant Shh components. Cilia are enriched in the smoothened (Smo), a Key component of the Shh signaling pathway [196], [197].

Smo in cilia enhance in a way the processing of Gli into activated forms. Gli then activates transcriptionally some target genes, such as Patched1 (Ptch1), becomes localized to the base of cilia [198]. Indeed, in response to Shh signaling, Ptch1 blocks cellular responsiveness to Shh by inhibiting Smo-dependent activation of the Gli effectors [199]. Conversely, when Hedgehog proteins bind to Ptch1, it diminishes its inhibitory influence on Smo, thus enhancing the activator forms of Gli proteins. As a result of the Gli transcriptional activity, several key target genes are strongly upregulated such as *FoxA2*, *Nkx2.2*, and *Olig2*, which specify floorplate, V3 interneuron, and motorneuron progenitor programs, respectively [200]. The mutual repressive transcription factor network limits the extent of Shh signaling, stabilize the cell fates within developing neural tissue, and refines dorsal-ventral domains of neural progenitor cells [201].

In conclusion, the prevailing view of molecular pathways and morphogen signaling in the developing spinal cord addressed that opposing concentration gradients of Shh ventrally, and Wnts and BMPs dorsally, are at the origin of specifying distinct cell fates along the dorsal-ventral axis (figure 23). The system appears to be finely-tuned, since a small change in morphogen concentration is sufficient to alter cell fates illustrating the complexity of morphogen dynamics in vertebrate patterning [201], [202].

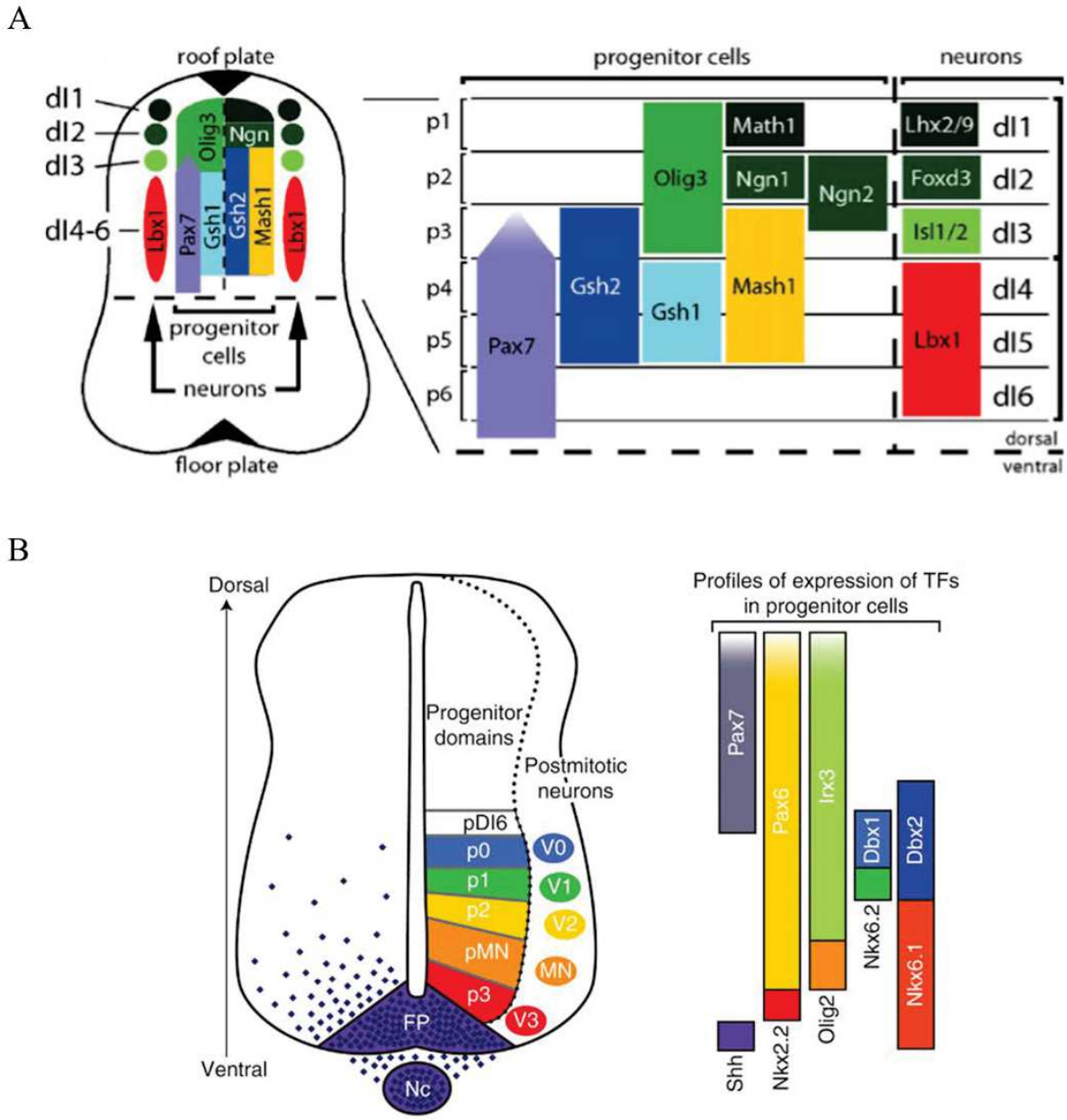


Figure 23: Expression of transcription factors and progenitor cells specification in the spinal cord. **(A)** Schematic dorsal representation of the six progenitor cell subpopulations, p1–p6, and their combinatorial expression of transcription factors of the bHLH and homeodomain families [170]. **(B)** Schematic ventral representation of the six domains of progenitor cells, FP, p3, pMN, p2, p1, and p0, which generate V0–V3 and MN neuronal subtypes, and their combinatorial expression of transcription factors [203].

Chapter V: Spinal cord injury

Spinal cord injury is the interruption of the spinal cord tissue, particularly it is the damage of axons and death of neurons. So it is a massive cell death and a loss of motor function and sensory inputs below the injury level. SCI affects CNS intrinsic neural cells (neurons, astrocytes, OPCs, ependymal cells), CNS intrinsic non-neural cells (microglia, pericytes, etc.) and immune cells from the blood and they all respond differently to an injury (figure 24) [204]. Upon SCI, the acute phase, is characterized by the immediate damage of spinal cord, which leads to the loss of both neurons and glial cells, including oligodendrocytes that should be remyelinating the surviving neurons. As a consequence, the loss of neurons leads to the dysfunction of the motor and sensory system. While during the sub-acute phase, microglial cells are activated in response to the production of free radicals, and secreted chemokines, and cytokines from ongoing necrotic neurons and glial cells [205]. In response to the activation, microglia and other inflammatory activated cells (macrophages, lymphocytes) infiltrate the lesion site for further inflammatory response that is accompanied by generation of free radicals that will kill the myelinating oligodendrocytes, thus keeping axons denuded and vulnerable, therefore a massive death of neurons takes place [205], [206]. Following the sub-acute phase of SCI, Wallerian degeneration, an ordered process of axonal death is undertaken [207]. In response to injury, the axonal skeleton of the nerve fiber disintegrates, and the axonal membrane breaks apart, causing axonal degeneration and release of myelin debris. Myelin debris has found to be the source of neurite outgrowth inhibitor A (Nogo-A), oligodendrocyte myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) that act as axonal regeneration inhibitors [208]. However, the slowly infiltrating immune cells and the low myelin clearing capacity of oligodendrocytes cause accumulation of myelin in the CNS tissue after injury. This accumulated myelin in turn participates in the apoptosis of oligodendrocytes and further contributes to the failure of remyelination and regeneration [205], [209].

5.1 Scar formation, Niche activation, and regenerative potential

SCI is directly followed by the formation of glial scar which is the hyper-activation of astrogliosis that could hold both positive and negative effects on the lesion repair (figure 24). Normally the glial scar could lead to permanent functional defects as it could cause cell death and disruption of spinal cord barrier. The scar is composed of both fibrotic and glial components that have beneficial functions from limiting tissue damage to preventing neuron loss. Fibrotic component is known to be in the center of the scar and composed of blood vessel–associated

type A pericytes derived somatic cells [158], [210]. The fibrotic scar was believed to be nonfunctional or it could participate in a way to axon regeneration [204], [211]. However, it has been studied recently and confirmed that the absence of type-A pericytes or ependymal cells derived astrocytes prevents the sealing of wound and therefore worsen SCI outcomes [211], [212]. So fibrotic scar is a key component for sealing injury where its absence cause tissue healing defects. On the other hand, 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 [213]. Rather, the glial scar is the generation of astrocytes from the ependymal cells and NSCs of spinal cord, as well as from self-duplicating astrocytes and upregulate the GFAP expression [50], [101]. This reactive astrogliosis may attenuates axonal regrowth, but it serves as a barrier to block inflammation and immune cell infiltration to the lesion and prevent further tissue damage, thus enhancing axon growth after damage [64], [214]. The debate about whether it attenuates or enhance axonal growth was solved by impairing the formation of glial scar by transgenic mouse models. This model blocks the cell cycle of ependymal cells generating astrocytes or kills proliferative astrocytes. These studies have shown that the significant loss of glial scar leads to worsened secondary injury to the tissue and the loss of axonal regeneration [64], [212].

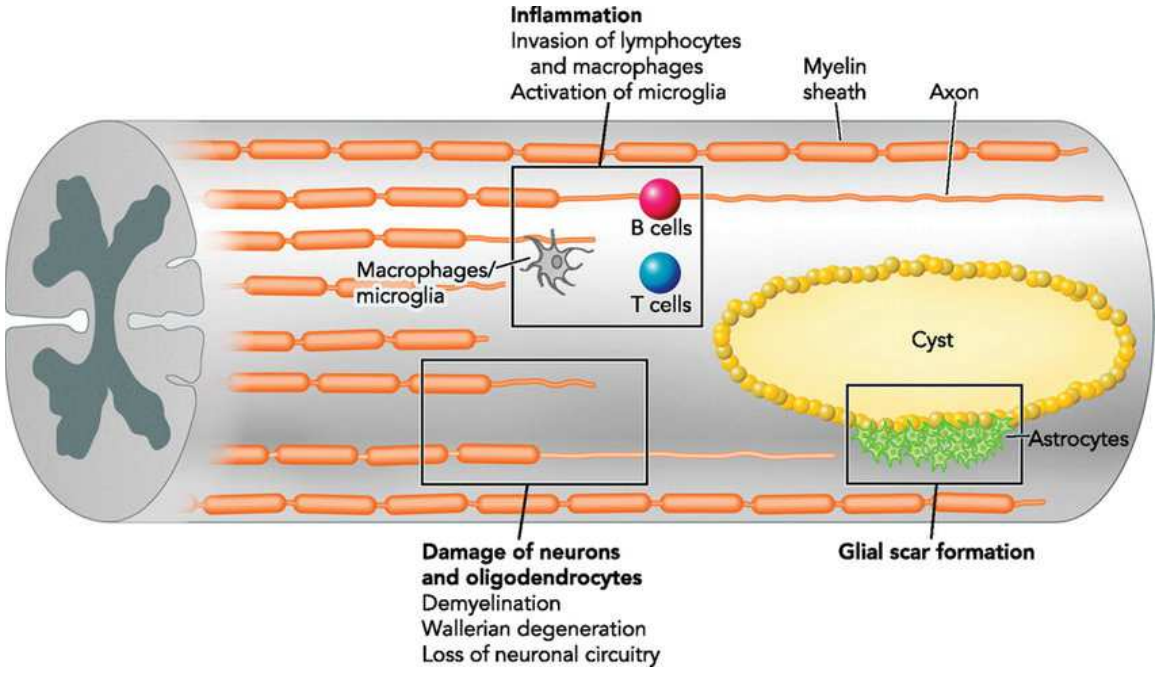


Figure 24: Schematic illustration of the responses in spinal cord in response to injury.
The diagram demonstrates the initiation of hypertrophic astrocytosis, invasion of inflammatory cells, and neuronal Wallerian degeneration [215].

In the spinal cord, it was shown long time ago that the ependymal cells have a high capacity to react directly to spinal cord injury. So it was interesting to study the activation of the niche and the response of ependymocytes to an injury, knowing that these two mechanisms differed among species. So it is interesting to highlight the differences in the ependymal neurogenic niche activity in response to SCI among regenerating and non-regenerating vertebrate species. Zebra fish represent a good regenerative model in vertebrates to understand the molecular mechanisms behind spinal cord regeneration. In physiological conditions zebra fish shows low or rare proliferation events and neurogenesis in the niche. However spinal cord lesion showed a high regenerative capacity, in which lesion induced activation of the niche in which ependymal radial glial (ERGs) cells activated, proliferated, migrated, and dedifferentiated. Finally these cells will exhibit a final differentiation step and generate motor neurons that integrate into the circuitry and muscle tissue (figure 25) [139], [216]. These data was confirmed by Michel M. Reimer when he used transgenic mice that targets ERGs and motor neurons through Olig2 and HB9 respectively. First his study showed that zebrafish has quiescent spinal progenitor cells in physiological conditions, and those cells got activated after injury. So the lesion in the spinal cord induced proliferation of ERGs where he noticed significant increase in the olig2+/BrdU+ cells around the stem cell niche. Furthermore, he confirmed by immunofluorescence the increase in motor neurons in the lesioned spinal cord represented by HB9-GFP+/BrdU+ cells. Finally he confirmed by the same assay that the newly generated motor neurons are derived from the proliferating ERGs represented by Olig2+/HB9+ where those cells are only present in the lesioned spinal cord but not in the intact one. The mechanism of spinal cord repair took only six weeks the period where proliferation and differentiation returns to normal levels. So the hallmark of motor neuron regeneration and spinal cord repair is the plasticity of quiescent Olig2+ ERGs after SCI [217].

Further studies confirmed the activation of the niche in zebra fish that showed a regenerative capacity after lesion. Of these studies the one launched by Kazuhiro Ogai where they depended on the use of the sex determining region Y-box2 (Sox2) gene to study its function and contribution to SCI repair in zebra fish. In fact this came from the observation of increased Sox2 expression in ependymal cells in zebrafish [137]. So they assessed the function of Sox2 following lesion, where data showed a significant up regulation of Sox2 in ependymal cells in response to injury. Interestingly the increased Sox2 expression preceded the proliferation of ependymal cells, where Sox2 knockdown caused decreased ependymal cell proliferation. Furthermore Sox2 up-regulation decreased GFAP reactivity around the canal an indication of ependymal dedifferentiation. Therefore Sox2 is one of the earliest transcription factor activated

in ependymal cells after SCI that act as an initiator of proliferation, and could be implicated in ependymal dedifferentiation [137], [218].

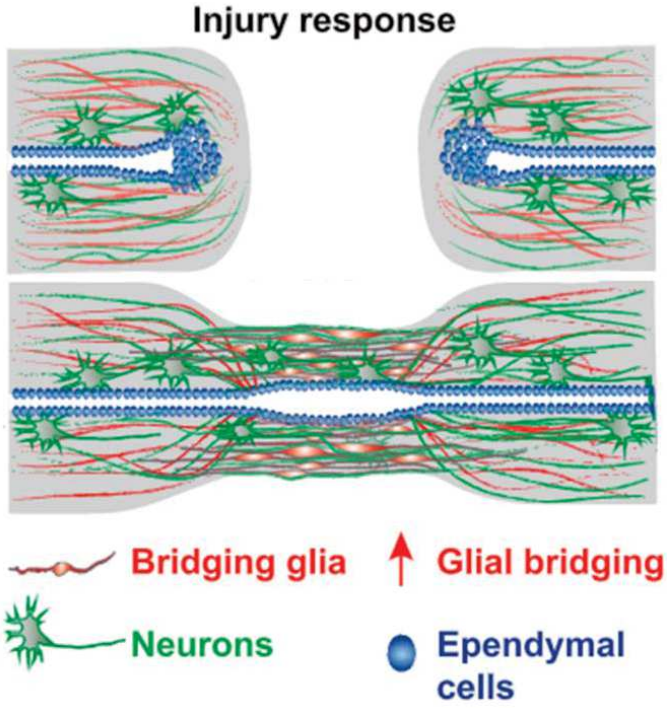


Figure 25: Schematic illustration of the multistep regeneration process in zebrafish spinal cord.
Spinal cord in zebrafish undergoes regeneration through activation, bridging, and remodeling [219].

The scenario of spinal cord repair differed in mammals from that of zebra fish. Although in mammals SCI cause permanent functional impairment, the NSCs activation or transplantation represent a promising tool for spinal cord repair. How the ependymal niche reacts to spinal cord injury?

Neural stem/progenitor cells, including ependymal cells, astrocytes, and OPCs are highly proliferative after SCI and display different cellular responses (Figure 26). Ependymal cells were found to be the only cell type displaying multipotency after SCI [50], [101]. Ependymal cells rarely divide around the central canal and can only generate a small number of neurospheres in cell culture under physiological condition. FoxJ1 (expressed in cells with motile cilia) and Nestin (expressed in stem/progenitor cells) are two transgenic mouse lines expressing tamoxifen-dependent Cre recombinase (CreER). These two lines restricted the expression to the cells lining the central canal, thus it allow the fate mapping of a subset of NSCs by targeting FoxJ1 and Nestin [50]. Using these mouse models they studied the response of ependymal cells to SCI. A lesion in the dorsal funiculus away from the ependymal layer, significantly increased the proliferation of ependymal cells. In addition to the massive

proliferation of recombined FoxJ1 and Nestin cells, there is a massive migration of these cells out of the ependymal layer. Recombined migrating cells lost FoxJ1 expression and lost their ependymal phenotype, instead they expressed Sox2, Sox3, Sox9, and the astrocytic marker GFAP. Also migrated cells where dependent from stromal cell-derived factor-1 (SDF1) and its major signaling receptor, CXCR4 and it is confirmed by using CXCR4-EGFP and SDF1-EGFP transgenic reporter mice. So the ependymal cells within the niche are activated after SCI and migrated due to SDF1/CXCR4 coupling which is present in these cells [220].

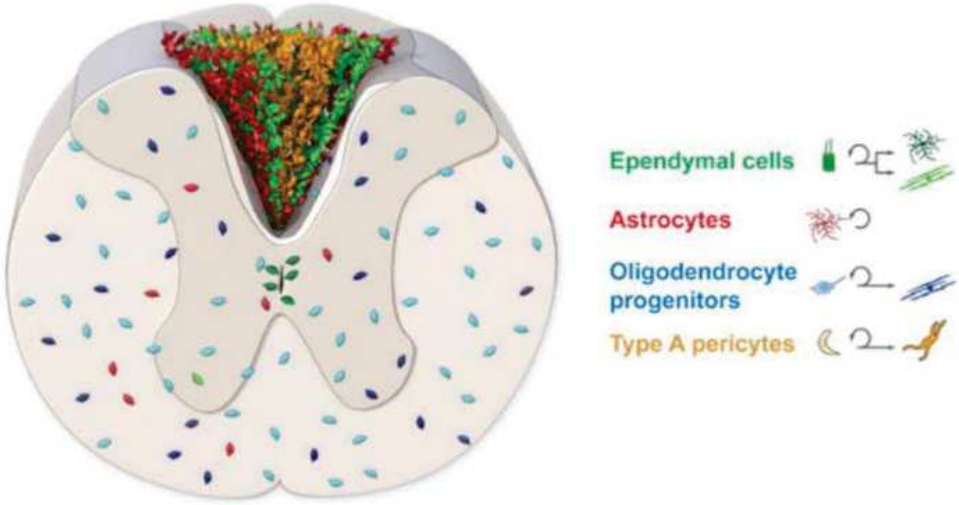


Figure 26: The response of endogenous cells after SCI.
Following a dorsal funiculus lesion, ependymal cells self-renew, differentiate, and the progeny migrate to the lesion site to form the glial scar with astrocytes (red) and pericytes (yellow) [65].

Following activation of the niche, ependymal cells contribute to glial scar formation (figure 24, and 26). Glial scar formation resulted from the migrating recombined cells expressing FoxJ1 and Nestin, and restricted to the injury site. Marker analysis of the ependymal progeny at the lesion site revealed that the majority of cells are sox9 and vimentin positive with astrocyte like morphology, and they colonize the center of the scar. On the other hand, there is a small subpopulation of recombined nestin cells that express the GFAP astrocytic markers and those cells were more close to the spinal cord surface and form the boarder of the scar. These results indicated that, the glial scar is a heterogeneous tissue made up of two astrocyte like cell populations, abundant sox9+/vimentin+ ependymal derived population and a GFAP+/nestin+ reactive resident astrocyte population [50]. It has been published that ependymal cells derived astrocytes are the main source of neurotrophic factors after SCI. NSC-derived cells synthesize several neurotrophic factors such as ciliary neurotrophic factor (CNTF), hepatocyte growth factor (HGF), and insulin growth factor (IGF-1). These factors are known to play a role in

neuron support, and their levels increase as Foxj1 proliferate after injury. So the glial scar formed by NSCs or astrocyte duplication they contribute positively to the injured tissue. Ablation studies of astrocytes showed massive infiltration of immune cells that enlarged the injury volume, and increased neuronal death [64].

Another event that takes place after SCI is the generation of oligodendrocytes by ependymal cells (figure 26) [101]. OPCs are the dividing cells in intact spinal cord and they increase after injury, to generate myelinating oligodendrocytes. Ependymal cell progeny showed oligodendroglial like ultrastructural morphology and marker profile. Further analysis on these cells showed that, ependymal-derived progenitors are located in the formed glial scar, where few Olig2+ cells are scattered in the grey and white matter bordering the injury. The Olig2+ cells displayed a morphology similar to that of mature oligodendrocytes. First they showed expression of mature markers such as MBP, second fine process were able to enwrap immunoreactive axons surrounding their myelin sheath. Moreover, ependymal cells from an injured spinal cord generate a significantly higher number of neurospheres in vitro and can be passaged 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 [120].

Furthermore, the inflammatory system is a potential regulator of ependymal cell responses to SCI. lesion to spinal cord will trigger resident NSCs to secrete proinflammatory signals that are maintained by activated immune cells and enhance the release of pro- and anti-inflammatory cytokines [221]. Microglial activation is associated with morphological changes and alteration in cytokine expression. Furthermore, lymphocytes are rapidly recruited to the lesion site by the activated and circulating neutrophils and monocytes-derived macrophages [222], [223]. After one weak of injury only macrophages and lymphocytes are present, and like astrocytes macrophages and microglia have dual effect, a protective beneficial role (protect and activate OPC proliferation) and a negative toxic role (induce cytotoxicity and demyelination) [224].

Early postinjury microglia and macrophages release Proinflammatory cytokines, such as tumor necrosis factor-a (TNFa), interleukin (IL) 1b, and IL-6. These factors are responsible for secondary tissue damage, modulating axonal remyelination, and stimulating astrogliosis. These changes are accompanied by the induction of adhesion molecules expression and the production of chemotactic factors, such as IL-8, monocyte chemotactic protein (MCP)-1, and macrophage inhibitory protein (MIP)-1a [225]. Studies on selective depletion of monocyte-derived

macrophages showed improved recovery of function, protected myelination, and stimulated sprouting and regeneration of axons following SCI [226].

On the other hand, neuroprotective molecules such as transforming growth factor (TGF)-b, and NT-3 are secreted by microglia/macrophages. The autocrine effect of TGF-b will inhibit microglia activation that blocks proinflammatory cytokine and reactive oxygen intermediate production, and together with BDNF and NT-3 they promote neuronal survival. The double function of microglial/macrophage population could be due to the existence of at least two subpopulations, M1 and M2, showing neurotoxic and proregenerative properties, respectively [227]. Shifting the polarization of these cells towards the M2 phenotype is a promising area of investigation [224]. It is unknown whether endogenous neural stem cells could be enhanced by modulating the levels of inflammation associated cells and their released factors after CNS injury.

To summarize up the similarities and differences between regenerating and non-regenerating species, first cells in the EZ of both species differ morphologically but they express transcription factors related to « stemness », such as Sox2. Second, the neuronal lineages of progenitor cells are fully expressed in zebrafish, while in mammals this potential is restricted by environmental factors. Third, EZ cells in both species massively proliferate in response to lesion signals after injury, where EZ cells are pushed out of their quiescence to start proliferating. Therefore, both species could share similar lesion induced intracellular mechanisms. However, differences in the activity levels of Notch and hedgehog could explain the difference in the net outcomes in terms of gliogenesis versus neurogenesis. Finally, these findings suggest that ependymal cells act as a scaffold to reinforce the injured spinal cord by restricting secondary enlargement of lesions [64], [117].

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 [228], [229]. It was reported that neural stem cell's potential decreases during aging after traumatic brain injury due to the quiescence but not the loss of neural stem cells, and also due to the change of their differentiation potential [230]. Indeed, astrocytes and ependymal cells significantly change their morphology and molecular signatures during brain aging, and their proliferation capacity decreases over time [231]. After SCI, markers of astroglial and inflammatory increase 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 [232].

Besides the changes in the microenvironment, the intrinsic regenerative potential of stem/progenitor cells is also age-dependent. At early age (P10), the spinal cord stem cell potential is entirely represented by ependymal cells, where their self-renewal capacity, and their differentiation capacity to oligodendrocytes significantly decreases at the juvenile stage and even more in adulthood in mice in both physiological and injured conditions (Figure 21, 27). Similarly, the recruitment and differentiation capability of OPCs in aged animals decline over time. These findings suggest that the decreased self-recovery capacity with increased aging is partly due to the decreased endogenous recruitment and remyelination potential by ependymal cells and APCs [156].

At early age, astrocytes, ependymal cells and type A pericytes are highly proliferative and pro-regenerative compared to adult stage, where these cells rapidly proliferate and contribute to the scar formation in both juvenile and adulthood in response to injury. However, following a SCI, juvenile ependymal cells are highly activated in vitro, showing greater self-renewal capacity and more oligodendrocyte differentiation, and higher sealing efficiency compared to adult cells [101], [233]. In comparison to adult lesions, juvenile lesions have a smaller fibrotic core and smaller glial scar, as well as less infiltration of microglia and blood-derived macrophages (Figure 27). Interestingly, even though ependymal cells have a higher stem cell potential in juvenile mice, their contribution to scar formation is lesion-severity dependent. They act as a backup reserve unless the lesion is large and more cells are need to be sealed [156]. Although ependymal cells are required for restricting enlargement of the lesion in adult [233], the same transgenic mouse model in which the cell cycle of ependymal cells is blocked showed a different phenotype in juvenile animals. Thus in the juvenile spinal cord other cell types could contribute to lesion sealing, while blocking ependymal cell proliferation does not lead to deeper lesions [156]. This suggest that the area of glial scar and the lesion core are also smaller even when ependymal cells are not able to proliferate. These data is in line with the clinical studies that have shown that juveniles have better functional recovery than adults in human [228].

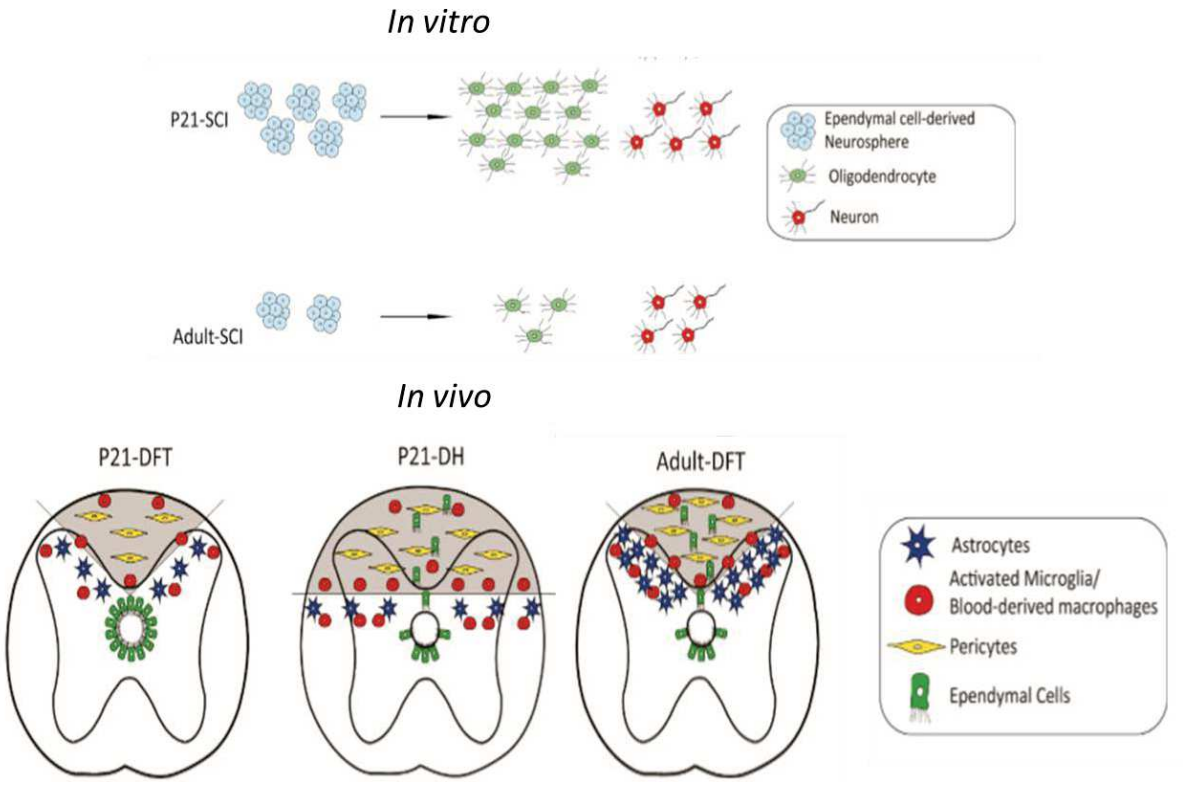


Figure 27: Ependymal cells and resident cells respond to SCI.
Under both physiological and injured conditions, ependymal cells show different self-renewal and differentiation capacity in juvenile mice compared to adult mice in vitro, and that the response is age- and lesion size-dependent in vivo [156].

5.2 Regenerative approaches

Deciphering the various scenarios occurring in the niche after a SCI, paved the way to better find a promising therapeutic potential for enhancing functional recovery. Regenerative strategies are many and could target several aspects in injury vicinity, it could be by (1) cellular replacement through transplantation, (2) modulating the injury microenvironment, or (3) modulating the injury response from endogenous cells.

1-Although there are a number of challenges for clinical applications, cell transplantation-based therapies have given some promising results for patients with SCI. Studies have focused on cellular replacement by transplanting NSCs to derive functional recovery after SCI as it caused massive loss and reduction in neurons and oligodendrocytes [234]. One of the most commonly used cell types for transplantation in SCI studies is MSCs that could be derived from bone marrow, umbilical cord, amniotic liquid and adipose tissue. 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),

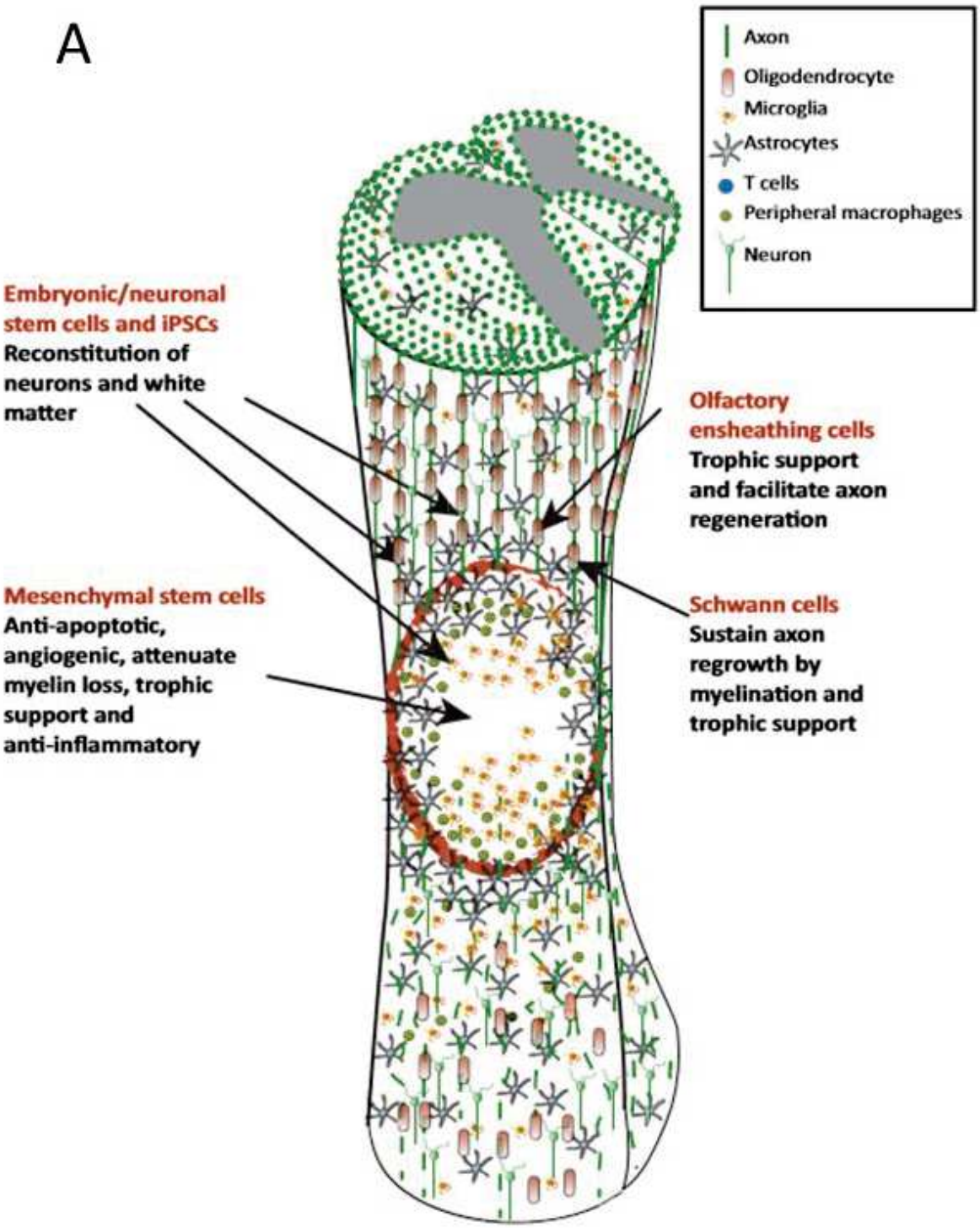
GDNF and BDNF [235]. The clinical score of American Spinal Injury Association, electromyography and magnetic resonance imaging in clinical trials have shown that SCI patients after MSC transplantations gain motor and sensory improvements [236], [237].

Embryonic stem cells (ESCs) are good candidate as it could compensate for the massive cell loss after SCI. ESCs can be obtained from embryonic tissues or clonally derived from ESC cultures. Animal studies showed that transplanted ESCs (figure 11) can differentiate into neurons [238] or oligodendrocytes [239] in SCI models, and promote significant motor functional recovery. Another group used a combination therapy of ESCs transplantation combined with docosahexanoic acid (DHA) treatment in the brain. In their study they indicated that NSCs transplantation into the injured cortex and DHA supplementation are promising therapies to treat TBI. The injected cells migrated to the injured area as well as to other brain regions implicated in motor activity. Furthermore, they demonstrated their effect on enhancing neurogenesis that is necessary for injury repair as well as motor recovery [66]. Recently, a new protocol showed that transplanting human ESC-derived neural stem cells into rats with SCI improved significantly the functional recovery as they differentiate into diverse neuronal and glial fates in vitro [240]. Despite the significant efficacy of ESC transplantation in preclinical studies, the ethical concerns with the uncontrollable side effect of teratoma formation limits the potential clinical applications of ESCs [241].

Although ependymal cells have a potential to generate neurons in culture, but are incapable of to do so after SCI, rather they generate astrocytes and oligodendrocytes. This is due to the fact that, the spinal cord environment favors gliogenesis over neurogenesis, thus pushing transplanted NSCs toward the gliogenic fate [50], [101]. However, when same cells are transplanted in the hippocampus neurogenic niche they differentiate into neurons [102]. Even though engineering NSCs to express pro-neural gene Neurogenin2 (Ngn2) generated glial cells after transplantation [242], thus the spinal cord environment is selective and highly restrictive for neurogenesis and neuron survival. However, Lu and colleagues, reported an increased neuronal differentiation, axonal growth and connectivity in the grafts and improved functional recovery by embedding NSCs transplants in fibrin matrices containing a cocktail of growth factors including BDNF, IGF-1, HGF, EGF and bFGF [67]. Although this study showed impressive data but it could be due to the administration of growth factors in the fibrin matrices. After what have been reported regarding the gliogenic nature of the adult spinal cord, it might be more interesting to modulate or enhance the generation of non-neuronal cells than to replace dying neurons to promote functional recovery.

Autologous glial cells specifically, olfactory ensheathing cells (OECs) transplantation also leads to improved outcomes after SCI. OECs which are located in the olfactory system can support and guide axonal growth from the peripheral nervous system to the CNS during development, as well as support adult neurogenesis or axonal regeneration. Therefore, OECs secrete many neurotrophic factors, such as BDNF, GDNF, VEGF, NT-3, etc. [243], thus they promote axonal regeneration, neuroprotective effects and functional recovery after being transplanted into the injured spinal cord [244]. As OECs are highly differentiated cells in comparison to ESCs, OEC transplantation upon SCI overcomes the risk of teretoma formation [245]. Interestingly, some studies showed the effect of olfactory bulb-OECs (bOECs) on ependymal cells using FoxJ1-CreERT2-YFP mouse. Transplantation of bOECs after SCI can enhance proliferation of ependymal cells in vivo and self-renewal capacity in vitro. Furthermore, bOECs trigger ependymal cells to differentiate into astrocytes, reduce the expression of axonal regrowth inhibitors, such as CSPGs and Neurocan, and promote adult neurogenesis after SCI. All together suggest that bOEC transplantation stimulates endogenous stem/progenitor cells, leading to beneficial effects on recovery of the injured spinal cord [246], [247]. The main strategies using cell transplantation therapies upon SCI are summarized in (figure 28 below).

2- Although cell transplantation has benefits on functional recovery, but this could be due to the modulation of the spinal cord microenvironment. Cell transplantation could replace lost cells and promote axonal regeneration through bridging the scar. This could not be due to the transplanted cells themselves, but rather due to the fact that, the environment is modulated in a beneficial way by the transplanted cells. For example transplanted mesenchymal cells secrete various factors such as BDNF, NGF, and VEGF that promotes axonal growth and survival where no cell replacement is observed [249]. The same functional recovery was observed after fibroblast transplantation or neurotrophic factors administration [250]. The administration of factors is known to increase sprouting, axonal growth, neuronal and/or oligodendrocyte survival, as well as it enhance proliferation of ependymal cells [250], [251].


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

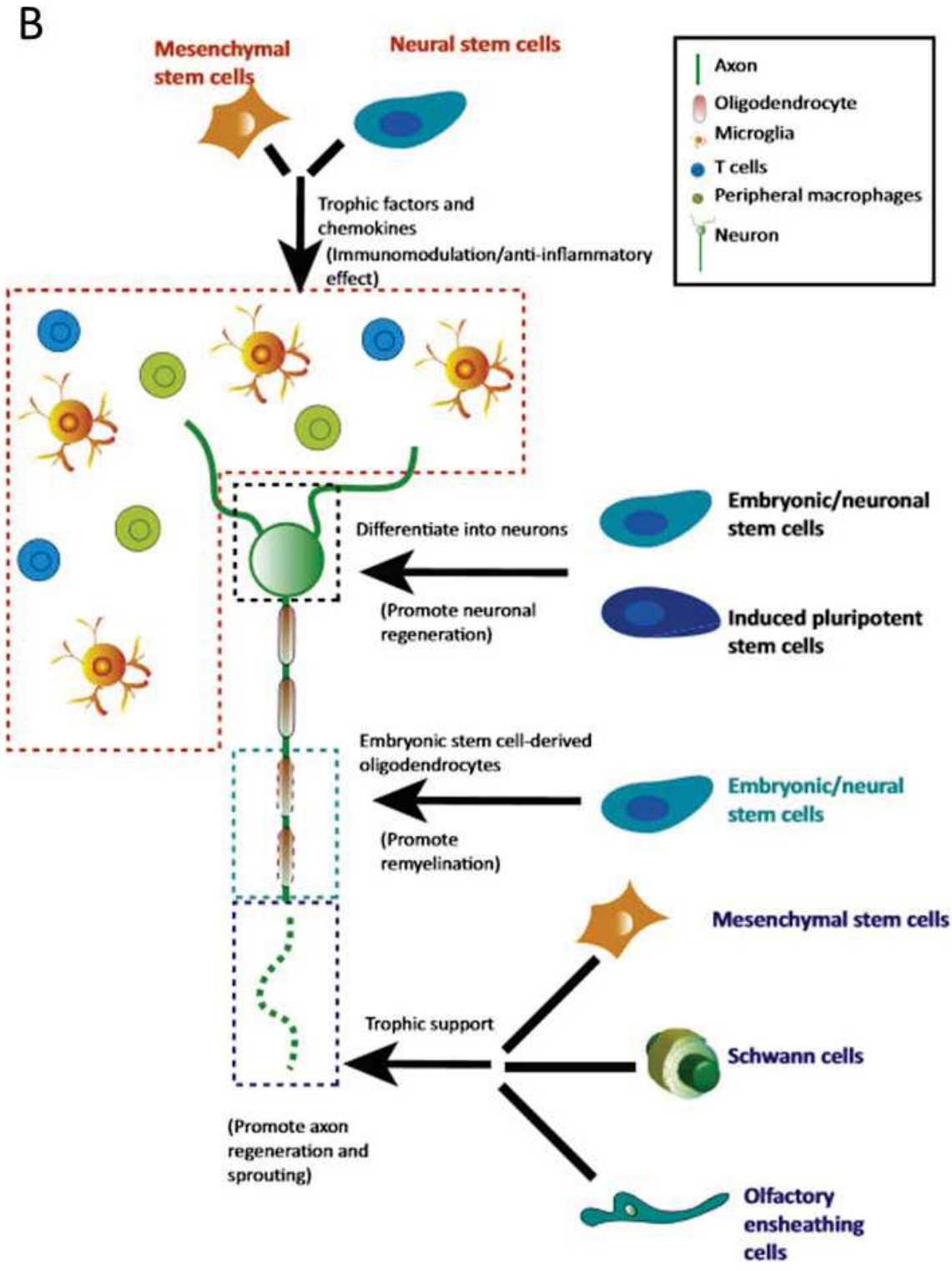


Figure 28: Schematic illustration of the main stem cell based transplantation.
(A) The cartoon illustrates the overview of transplanted stem cells in injured spinal cord. **(B)** The cartoon illustrates a detailed view of transplanted stem cells inside the injured spinal cord showing the detailed interplay driving anti-inflammatory and proregenerative processes on neuronal cytoarchitecture in SCI [248].

3- Modulating the injury response from endogenous cells is triggered by three ways. First, modulation of the astrocytic contribution to the glial scar. It appears that the astrocyte population contains two subtypes a growth-promoting and a growth-inhibiting ones [233], [252]. Reactive astrocytes prevent axon growth and limit the regeneration due to the expression of inhibitory factors, such as chondroitin sulfate proteoglycans [253], [254]. In contrast to this, several studies demonstrated the beneficial effects of the glial scar in tissue repair [204], [212]. Faulkner and colleagues showed that, by eliminating reactive astrocytes, the massive infiltration of inflammatory cells, thus a larger lesion volume and increased neuronal loss [253], [254]. Other studies showed that deleting the protein suppressor of cytokine signaling 3 (Socs3) in Nestin-positive cells (including ependymal cells) enhanced astrocyte reactivity, migration, scar formation, and functional recovery [255], [256]. Indeed, recent studies showed that spinal cord astrocytes are very phenotypically and functionally heterogeneous in the CNS and after SCI [213]. Blocking the subtype of astrocytes derived from ependymal cells after SCI for instance, leads to a more severe lesion and worsen functional recovery [233]. Combined together, these data report that the scar formation inhibits inflammatory response thus induce functional recovery.

Second, ependymal cells respond to injury by enhancing the differentiation of oligodenndrocytes, since oligodendrogenesis can enhance functional recovery [65], [233]. Following an injury, a zone of unrepaired demyelination appears, in this manner both OPCs and ependymal cells can generate oligodendrocytes in response to injury. Ependymal-derived oligodendrocytes represent 3% of the total ependymal progeny [101]. The low percentage of ependymal-derived oligodendrocytes is due to the massive secretion of astrocytes-promoting factors such as IL-6 related cytokines and BMPs. Supporting the idea of stimulating oligodendrocyte generation from spinal cord ependymal cells, neurospheres overexpressing neurogenin-2 are more capable of generating oligodendrocytes, enhance myelination, and enhanced motor and sensory functional recovery when transplanted into injured spinal cord [242]. So from a clinical point of view, it is interesting to induce oligodendrocytes production from endogenous ependymal cells. This will be a strategy to avoid invasive cell transplantation and enhance immunosuppression. However it is not known if this strategy can overcome the effect of astrocytes-promoting environment.

Third, modulating injury response from endogenous cells through enhancing neuronal differentiation is one of the strategies that could be used. The major cause of functional deficits after an injury is the loss of neurons. So providing neuronal substrate could stimulate

neurogenesis and integration of neurons into the circuitry and could promote functional recovery through electrical signals that bridge the lesioned area [257].

Over the past years several trial were taken into consideration to overcome the challenges behind treating SCI by endogenous NSCs. So in this manner studies have proposed two novel functions of ependymal cells that are latent NSCs in the spinal cord. First, they have a scaffolding function by preventing the enlargement of the injured area by restricting the secondary injury after the initial damage. Second, ependymal cells supply neurons with the necessary neurotrophic factors to prevent them from dying in the spinal cord toxic environment. Also it has been proven that glial scar contributes positively into the SCI, where they identified the mechanisms that could be targeted to modulate responses to injury and enhance recovery [65], [212], [233].

OBJECTIVES

A key question one could ask, why mammalian spinal cord do not regenerate? And why evidence of neurogenesis is still abscent? To answer those questions it is interesting first to understand the mammalian spinal cord environment particularly the endougenous stem cell niche environment that is the source of repair in regenerative species. However the various cell types that reside in the niche are not yet well identified in both mouse and human. Moreover the signaling pathways that regulate the quiescence and activation of the niche are ill defined, and whether these pathways are involved in spinal cord patterning during development are regulating the nich in the adult. To have a clear view on the differences illustrated in the introduction about regenerative and non-regenerative species and to understand the difference between mouse and human spinal cord niche, a comparative analysis is needed to unravel the differences, finally it is interesting to mimic the regenerative models by stimulating the stem cell niche and look for regenerative evidences. The goal of this thesis is to study deeply the genetic signature of the human and mouse spinal cord stem cell niche in intact and injured spinal cord. Also trying to generate a new stem cell niche model representing all cell types that resides in the niche and their characteristics. Interestingly, to identify the transcription factors expressed by the cells and the molecular pathways that regulate them. The specific aims are:

- To unravel the genetic signature of the central canal niche in intact and injured spinal cord;
- To identify the subtypes of cells residing in the canal and their response to spinal cord injury;
- To characterize the identity of peri-neuronal cells in the spinal cord.

RESULTS

Part I

The spinal cord central canal: A diverse regionalized stem cell niche

Publication 1

Adult human and mouse spinal cord ependymal region maintain an embryonic-like dorsal-ventral regionalization with dorsal Msx1+ neural stem cells

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001

Stem Cell Reports
Resource
ISSCR
OPEN ACCESS

RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1$^+$ Roof-Plate-Derived Cells

Hussein Ghazale,[1,9] Chantal Ripoll,[1,9] Nicolas Leventoux,[1] Laurent Jacob,[2] Safa Azar,[1] Daria Mamaeva,[1] Yael Glasson,[1] Charles-Felix Calvo,[3] Jean-Leon Thomas,[2,4] Sarah Meneceur,[5] Yvan Lallemand,[5] Valérie Rigau,[1,6] Florence E. Perrin,[8] Harun N. Noristani,[8] Brenda Rocamonde,[2] Emmanuelle Huillard,[2] Luc Bauchet,[1,7] and Jean-Philippe Hugnot[1,8,*]

[1]INSERM U1051, INM, Hopital Saint Eloi, 80 Avenue Augustin Fliche, 34091 Montpellier, France
[2]Inserm U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière, ICM, 75013, Paris, France
[3]Neuroglial Interactions in Cerebral Physiopathology. CIRB, CNRS UMR 7241/INSERM U1050 Collège de France 11, Place Marcelin Berthelot, 75005 Paris, France
[4]Department of Neurology, Yale University School of Medicine, New Haven, CT 06511, USA
[5]Institut Pasteur, Department of Developmental and Stem Cell Biology, UMR3738 CNRS, Paris 75015, France
[6]CHU of Montpellier, Hopital Gui de Chaulliac, Pathology Department, 80 Avenue Augustin Fliche, 34091 Montpellier, France
[7]CHU of Montpellier, Hopital Gui de Chaulliac, Neurosurgery Department, 80 Avenue Augustin Fliche, 34091 Montpellier, France
[8]University of Montpellier, Faculté des Sciences, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France
[9]Co-first author
*Correspondence: jean-philippe.hugnot@umontpellier.fr
https://doi.org/10.1016/j.stemcr.2019.04.001

SUMMARY

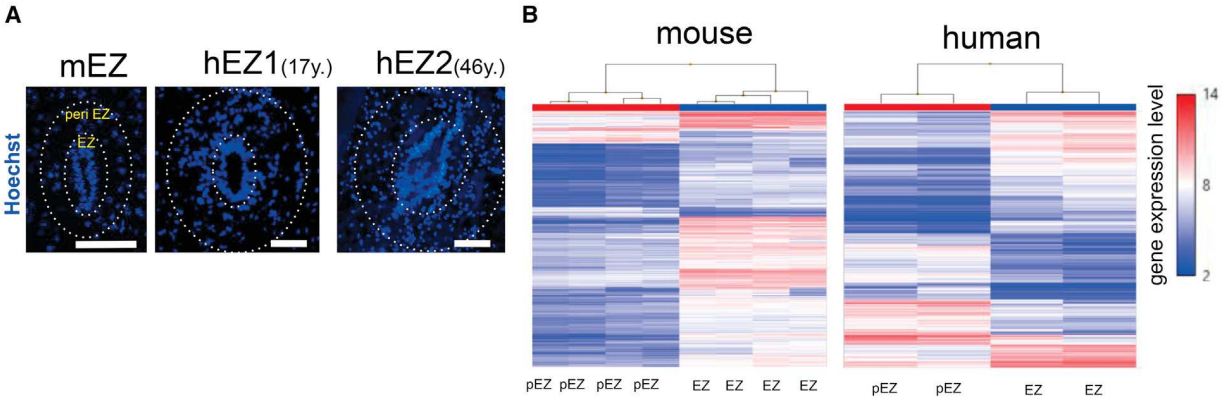
Anamniotes, rodents, and young humans maintain neural stem cells in the ependymal zone (EZ) around the central canal of the spinal cord, representing a possible endogenous source for repair in mammalian lesions. Cell diversity and genes specific for this region are ill defined. A cellular and molecular resource is provided here for the mouse and human EZ based on RNA profiling, immunostaining, and fluorescent transgenic mice. This uncovered the conserved expression of 1,200 genes including 120 transcription factors. Unexpectedly the EZ maintains an embryonic-like dorsal-ventral pattern of expression of spinal cord developmental transcription factors (ARX, FOXA2, MSX1, and PAX6). In mice, dorsal and ventral EZ cells express *Vegfr3* and are derived from the embryonic roof and floor plates. The dorsal EZ expresses a high level of *Bmp6* and *Gdf10* genes and harbors a subpopulation of radial quiescent cells expressing MSX1 and ID4 transcription factors.

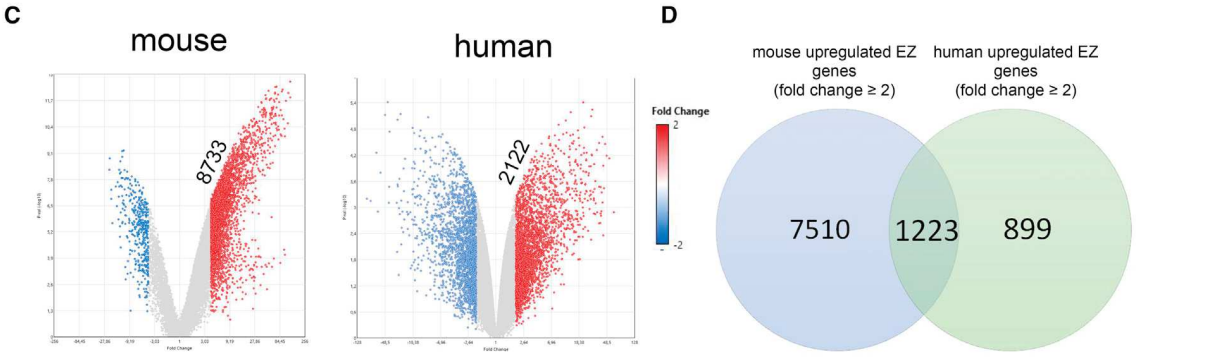
INTRODUCTION

The adult central nervous system maintains neural stem cells in specific areas called niches (Gage and Temple, 2013). The main stem cell pools are in the subventricular zone (SVZ) and in the hippocampus. A third stem cell niche is found in the adult spinal cord around the central canal in anamniotes, rodents, and humans (Becker et al., 2018; Marichal et al., 2017; Stenudd et al., 2015). This niche originates from the embryonic neuroepithelium and forms the spinal cord ependymal zone (EZ) organized as a pseudo-epithelium. As in the brain, this stem cell niche is highly organized and contains stem and non-stem cells. Four different cell types, namely ependymocytes, cerebrospinal-fluid-contacting neurons (CSF-N), vessels, and long radial cells have been described in mice. In particular, the EZ presents a dorsal-ventral regionalization with long radial glial fibrillary acidic protein (GFAP)$^+$ cells in the dorsal part (Sabourin et al., 2009). In contrast to brain, spinal cord ependymal cells slowly proliferate to self-renew (Alfaro-Cervello et al., 2012; Pfenninger et al., 2011). In cultures, a fraction of these cells can also generate passageable neurospheres (i.e. clonal expansion of neural precursor cells) which can generate astrocytes, oligodendrocytes, and neurons after differentiation (Weiss et al., 1996). The identity of these neurosphere-forming cells in the EZ is still not completely clear as both GFAP$^+$ and GFAP$^-$ ependymal cells can behave as neural stem cells *in vitro* (Barnabé-Heider et al., 2010; Fiorelli et al., 2013; Sabourin et al., 2009; Xu et al., 2017). Recent single cell analysis has identified neurogenesis in the adult spinal cord (Habib et al., 2016); however, whether these new neurons are derived from the EZ is not yet established. It has been known since 1962 (Adrian and Walker, 1962) that the EZ can readily activate and produce new cells upon injury (Becker et al., 2018). Depending on the lesion type and severity, EZ-derived cells can significantly contribute to the glial scar formation (Ren et al., 2017; Stenudd et al., 2015).

In comparison with the brain niches, less is known about the adult spinal cord EZ. Reminiscent of the mouse niche, in human, ependymal cells around the central canal display immature features such as expression of NES (nestin), VIM (vimentin), and SOX2 (Becker et al., 2018). However, with aging the central lumen can disappear and the EZ is disorganized (Garcia-Ovejero et al., 2015). Multipotent neurospheres with a limited proliferation ability

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001





E

Transcription factors ($p< 2.5\ 10^{-7}$)

AHCTF1	FOXJ1	KLF5	NFATC2	PIAS1	SMAD5	ZFP3
ARX	GABPA	MDFIC	NFIA	POU2F1	SOX11	ZFPM2
ATF1	GLIS3	MED1	NFIB	POU3F2	SOX2	ZFX
BACH2	GPBP1	MED13L	NFIX	PRDM5	SOX4	ZHX2
BCL10	HDGF	MED14	NFXL1	PSIP1	SOX5	ZKSCAN7
BCOR	HES1	MED17	NFYB	PTPN14	SOX6	ZKSCAN8
BPTF	HMGB2	MED25	NOTCH1	PURB	SOX9	ZSCAN18
CENPF	HOXA2	MED30	NR1D2	REST	SP4	
CITED2	HOXA3	MED31	NR2C1	RFX2	SPEN	
CREBBP	HOXB3	MEIS2	NR2C2	RFX3	TAF1	
CREBRF	ID4	MINA	NR2F1	RFX4	TAF1B	
CREBZF	IKZF2	MLXIP	NR2F2	RORA	TBL1X	
CRY1	IKZF4	MSX1	NR4A3	RREB1	TCF7L1	
CTNNB1	IRF2	MTA1	NRF1	RUNX1	TCF7L2	
DDX20	IRF6	MTA3	PAX6	SALL1	TEAD1	
E2F5	KAT6A	MTF1	PAXBP1	SALL3	TRPS1	
E2F7	KCTD1	MYB	PBX1	SATB1	TRRAP	
EP300	KDM3A	NCOA3	PER2	SIN3A	UBN2	
FOXA1	KDM5C	NFATC1	PHF21A	SMAD2	YAP1	

Cilia ($p< 4.6\ 10^{-52}$)

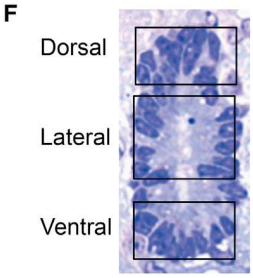
ARL13B	CEP164	IFT52	TTC8
B9D1	CEP290	IFT57	TMEM107
BBIP1	CEP41	IFT74	TMEM138
BBS1	CEP83	IFT80	TMEM17
BBS2	CFAP221	IFT81	TMEM216
BBS4	CFAP54	IFT88	TMEM231
BBS5	CC2D2A	INTU	TMEM237
DZIP1L	CCDC113	NOTCH1	UNC119B
DZIP1	DNAAF1	PCM1	
FBF1	DNAH5	RFX2	
IQUB	DYNC2H1	RFX3	
NEK1	FAM161A	RPGR	
OFD1	FOXJ1	SCLT1	
RILPL2	HSPB11	SPAG16	
TBC1D32	IFT122	TCTN1	
WDR19	IFT172	TCTN3	
WDR35	IFT20	TEKT3	
CEP126	IFT43	TTC21A	
CEP162	IFT46	TTC26	

Division ($p< 1.4\ 10^{-3}$)

CDC14A	NEK2
CDK13	NEK4
CDK6	NUDC
CDK7	NUMA1
CENPF	NUP37
CEP164	PARD3B
CETN2	PARD6B
CETN3	PARD6G
CHEK2	PSRC1
CIB1	RUVBL1
CKS1B	SMC1A
CNTRL	SMC3
EVI5	SPDL1
KATNA1	SPICE1
KATNB1	STAG2
LATS1	STOX1
MAD2L1	TTC28
NEDD1	
NEK1	

Smo. ($p<5.5\ 10^{-10}$)

ARL13B
B9D1
DZIP1
IQUB
CLUAP1
CC2D2A
DYRK2
HSPB11
HES1
HIPK1
IFT172
IFT20
IFT27
IFT46
IFT52
IFT57
IFT80
PAX6
TCTN3



G

VENTRAL EZ ENRICHED GENES

fold change ≥ 4.0

Gene Symbol	Gene description	Fold change Ventral vs Lateral	pval
Sulf1	sulfatase 1	14,4	0,03
Ucma	associated	11,7	0,03
Arx	aristaless related homeobox	11,5	0,03
Dcn	decorin	9,2	0,03
Rbp4	retinol binding protein 4, plasma	8,0	0,03
Sulf1	sulfatase 1	6,1	0,03
Foxa1	forkhead box A1	4,7	0,03
Prss23	serine protease 23	4,7	0,03
Fgl2	fibrinogen-like protein 2	4,3	0,03

DORSAL EZ ENRICHED GENES

fold change ≥ 2.0

Gene Symbol	Gene description	Fold change Dorsal vs Lateral	pval
A2m	alpha-2-macroglobulin	5,8	0,03
Gdf10	growth differentiation factor 10	5,3	0,03
Gcnt1	glucosaminyl (N-acetyl) transferase 1	3,3	0,03
Bmp6	bone morphogenetic protein 6	3,0	0,03
Efnb3	ephrin B3	2,6	0,03
Prokr2	prokineticin receptor 2	2,5	0,03
Dclk1	doublecortin-like kinase 1	2,2	0,03
Thbs2	thrombospondin 2	2,0	0,03
Hexb	hexosaminidase B	2,0	0,03

(legend on next page)

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001

have been derived from the human spinal cord (Dromard et al., 2008) and using alternative culture conditions, Mothe et al. (2011) were able to maintain a sustained proliferation of multipotent human-derived neural stem cells.

A detailed transcriptomic profiling of the human and mouse EZ is currently lacking. This would help us to understand the specificity and diversity of these cells as well as identify gene expressions and molecular pathways conserved between primates and rodents. It would also provide important insights into why, in contrast to anamniotes, mammalian ependymal cells cannot regenerate neurons after spinal cord injury (Becker et al., 2018).

Here we provide a cellular and molecular resource for the mouse and human EZ based on RNA profiling, immunostaining, and fluorescent transgenic mice. This uncovered the conserved expression of 1,200 genes specifically expressed in the EZ, including 120 transcription factors (TFs). Unexpectedly, the EZ maintains an embryonic-like dorsal-ventral pattern of expression of spinal cord developmental TFs. New subpopulations of cells expressing specific genes were identified in the dorsal and ventral part of the EZ. In mice, dorsal ependymal cells were found to be derived from the embryonic spinal cord roof plate.

RESULTS

Genes Enriched in the Adult Human and Mouse EZ

An epithelial organization of the EZ is observed both in human and mouse as evidenced by CTNNB1 (β-catenin) and CD24 stainings (Figures 2 and 3). To identify gene expression enriched in the EZ, we microdissected this region and adjacent tissue in two human samples and four mice (Figure 1A). For human, we selected two samples with a lumen from patients aged 17 and 46 years. Microarrays were used for RNA profiling and heatmaps indicated adequate clustering of EZ samples (Figure 1B). Volcano plots showed 8,733 and 2,122 genes enriched (fold change $\geq$2) in the mouse and human EZ, respectively, and 1,223 genes commonly enriched in both species (Figures 1C and 1D). Table 1 shows the top 15 genes commonly enriched in the human and mouse EZ and genes that are more specifically enriched in human or mice. Figure S1A shows examples of identified genes whose specific expression in the mouse EZ is confirmed in the Allen brain atlas (Lein et al., 2007). In mouse and human, GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses revealed an enrichment for genes involved in cilia formation, smoothened and hippo pathways, cell division, and transcription (Figure 1E and Tables S1, S2, and S3). In addition, consistent with their position at the interface between CSF and the nervous parenchyma, ependymal cells also expressed 129 and 34 members of the solute carrier family in mice and human, respectively (Tables S1, S2, and S3). Some of them are very specifically (fold change >30) expressed in the mouse EZ such as *Slc26A3*, *Slc14a1*, and *Slc16a12*, which transport chloride, urea, and monocarboxylic acid, respectively. Unexpectedly, *Cftr*, a chloride transporter responsible for cystic fibrosis, was found to be specifically expressed in the EZ in both species (Table 1). Using a single cell transcriptome approach in the mouse spinal cord, two recent studies (Rosenberg et al., 2018; Zeisel et al., 2018) provided a limited number of genes (<50) enriched in spinal cord ependymal cells and CSF-N, and most of them were identified in our study (Table S4). Interestingly, only *Tmem212*, a gene coding for a transmembrane protein with few annotations, was identified in the three studies (including ours) as being enriched in the ependymal cells. With regard to CSF-N, *Espn* (*Espin*) and *Pkd2l1* were identified in the three studies (Table S4).

We and others have reported that the dorsal and ventral parts of the mouse EZ have distinctive features such as the presence of radial cells expressing NES protein (Alfaro-Cervello et al., 2012; Becker et al., 2018; Hamilton et al., 2009) and the preferential expression of the TF ZEB1 in the dorsal part (Sabourin et al., 2009). Further exploration of regional gene expression was done by microdissecting the dorsal, lateral, and ventral parts of the mouse EZ (Figure 1F, n = 4 mice). Only few regionally-expressed genes were identified (Figure 1G and Table S5). In the ventral part, we found a strong expression of three genes

Figure 1. RNA Profiles of the Mouse and Human EZ

(A) Aspect of EZ in the mouse (mEZ, thoraco level) and human samples (hEZ1 and hEZ2, thoracolumbar level). A lumen was present in mice and in the two human (aged 17 and 46 years) samples. Microdissected EZ and peri EZ regions are delimited with dotted circles. Scale bars, 100 μm.

(B) Heatmap of hierarchical clustering of genes expressed in EZ and peri EZ (pEZ) regions in the four mouse and two human samples.

(C) Volcano plots of genes whose expression is enriched in the mouse and human EZ (fold change $\geq$2).

(D) Venn diagram of genes enriched in the mouse and human EZ.

(E) Genes enriched for ciliogenesis, smoothened pathway (Smo.), division, and transcription factors in the mouse and human EZ. Expression of genes in red was subsequently confirmed at the protein level (Figures 2 and 3).

(F) Microdissected subregions of the mouse EZ.

(G) Genes enriched (top 9) in the ventral and dorsal EZ (full lists are in Table S5).

Table 1. Three Lists of Genes (Top 15) Enriched in Mouse, Human, and Mouse and Human EZ

Top 15 Human EZ-Specific Genes						Top 15 Mouse EZ-Specific Genes					
Gene	Description	hEZ (log_2)	Peri hEZ (log_2)	Fold Change	p Value	Gene	Description	mEZ (log_2)	Peri mEZ (log_2)	Fold Change	p Value
C7orf57	chromosome 7 open reading frame 57	10.9	4.9	61.8	0.0013	Rsph4a	radial spoke head 4 homolog A	10.9	3.8	133.1	2.32×10^{-13}
VWA3B	von Willebrand factor A domain-containing 3B	11.4	5.7	52.8	7.43×10^{-5}	1500015O10Rik	RIKEN cDNA 1500015O10 gene	11.1	4.0	132.7	1.38×10^{-12}
NEK5	NIMA-related kinase 5	11.1	5.5	47.9	6.43×10^{-5}	Stoml3	stomatin (Epb7.2)-like 3	10.4	3.5	121.7	8.47×10^{-13}
CD36	CD36 molecule	10.5	5.0	45.8	3.50×10^{-5}	1700007K13Rik	RIKEN cDNA 1700007K13 gene	11.1	4.2	120.3	2.66×10^{-11}
EFHB	EF-hand domain family, member B	11.2	5.7	44.0	0.0004	Slc26a3	solute carrier family 26, member 3	11.2	4.4	110.5	2.05×10^{-12}
MYLK3	myosin light-chain kinase 3	11.4	6.0	42.6	0.0044	Tnnc2	troponin C2, fast	10.1	3.4	105.2	8.39×10^{-5}
CCDC39	coiled-coil domain-containing 39	10.0	4.6	42.4	0.0001	2810047C21Rik1	RIKEN cDNA 2810047C21	9.8	3.2	103.2	4.85×10^{-11}
ODF3B	outer dense fiber of sperm tails 3B	9.9	4.5	41.5	0.0008	Chil3; Chil4	chitinase-like 3; chitinase-like 4	11.5	4.8	102.2	4.87×10^{-5}
ZBBX	zinc finger, B-box domain-containing	10.6	5.3	41.3	2.38×10^{-5}	C1qtnf3	C1q and tumor necrosis factor related protein 3	10.7	4.1	97.9	1.63×10^{-11}
CCDC114	coiled-coil domain-containing 114	11.3	6.0	40.3	0.0005	Cfap161	cilia- and flagella-associated protein 161	10.5	3.9	95.3	5.65×10^{-13}
FAM216B	family with sequence similarity 216, member B	9.9	4.5	39.8	0.0012	Fam183b	family with sequence similarity 183, member B	11.5	4.9	91.8	1.06×10^{-11}
CFAP43	cilia- and flagella-associated protein 43	11.3	6.0	39.1	0.0003	Gm11992	predicted gene 11.992	9.8	3.3	91.6	7.02×10^{-13}
FMO3	flavin-containing monooxygenase 3	9.9	4.6	38.9	0.0014	Iqca	IQ motif containing with AAA domain	11.0	4.5	90.4	1.12×10^{-10}

(*Continued on next page*)

Table 1. *Continued*

Top 15 Human EZ-Specific Genes						Top 15 Mouse EZ-Specific Genes					
Gene	Description	hEZ (log_2)	Peri hEZ (log_2)	Fold Change	p Value	Gene	Description	mEZ (log_2)	Peri mEZ (log_2)	Fold Change	p Value
CFAP70	cilia- and flagella-associated protein 70	11.3	6.0	38.5	0.0023	Capsl	calcyphosin-like	9.6	3.1	87.3	5.95×10^{-12}
SPATA17	spermatogenesis-associated 17	10.3	5.1	37.1	0.0003	Acta1	actin, alpha 1, skeletal muscle	10.7	4.2	85.5	0.0002

TOP15 Human and mouse EZ-specific genes

Gene	Description	hEZ (log_2)	peri hEZ (log_2)	Fold Change	p Value	mEZ (log_2)	peri mEZ (log_2)	Fold Change	p Value
CCDC39	coiled-coil domain containing 39	10.0	4.6	42.4	0.0001	9.6	3.5	68.5	9.13×10^{-12}
ODF3B	outer dense fiber of sperm tails 3B	9.9	4.5	41.5	0.0008	10.5	4.4	68.7	1.01×10^{-10}
RSPH4A	radial spoke head 4 homolog A (Chlamydomonas)	9.8	4.9	30.0	0.0004	10.9	3.8	133.1	2.32×10^{-13}
ARMC4	armadillo repeat containing 4	10.3	5.1	36.7	3.27×10^{-5}	9.9	4.1	58.9	9.43×10^{-12}
CAPSL	calcyphosine-like	11.3	6.4	31.0	0.0001	9.6	3.1	87.3	5.95×10^{-12}
MNS1	meiosis-specific nuclear structural 1	11.0	6.1	30.7	0.0014	10.4	4.2	72.3	3.96×10^{-13}
DYNLRB2	dynein, light chain, roadblock-type 2	11.2	6.2	30.6	0.0007	11.3	5.1	72.4	1.63×10^{-12}
TTC29	tetratricopeptide repeat domain 29	9.7	4.9	28.4	6.06×10^{-5}	9.5	3.4	67.9	8.98×10^{-13}
TEKT1	tektin 1	9.8	5.0	27.3	0.0004	10.1	4.1	64.7	2.59×10^{-11}
WDR63	WD repeat domain 63	10.0	5.3	26.3	0.0005	9.6	3.5	66.4	2.21×10^{-12}
ARMC3	armadillo repeat containing 3	9.7	5.0	26.0	0.0002	9.1	2.9	73.2	4.72×10^{-11}
ZMYND10	zinc finger, MYND-type containing 10	9.0	4.5	23.5	0.0002	9.8	3.8	62.8	5.05×10^{-12}
STOML3	stomatin (EPB72)-like 3	9.3	4.8	23.4	7.92×10^{-5}	10.4	3.5	121.7	8.47×10^{-13}
SPEF2	sperm flagellar 2	9.3	4.8	22.0	1.46×10^{-5}	7.2	3.2	15.6	2.52×10^{-9}
CFAP52	cilia and flagella associated protein 52	12.0	7.6	21.3	0.0013	10.6	4.3	81.3	1.07×10^{-11}

Top 15 Human and Mouse EZ Transcription Factors

Gene	Description	hEZ (log_2)	Peri hEZ (log_2)	Fold Change	p Value	mEZ (log_2)	Peri mEZ (log_2)	Fold Change	p Value
ARX	aristaless-related homeobox	8.6	4.6	16.4	0.0012	6.7	3.3	10.6	2.15×10^{-9}
RFX2	regulatory factor X, 2	9.6	5.6	16.2	0.0018	7.6	3.5	17.3	5.10×10^{-8}

(*Continued on next page*)

Table 1. *Continued*

Top 15 Human and Mouse EZ Transcription Factors

Gene	Description	hEZ (log_2)	Peri hEZ (log_2)	Fold Change	p Value	mEZ (log_2)	Peri mEZ (log_2)	Fold Change	p Value
SOX6	SRY box 6	10.4	8.3	4.5	0.0016	9	5.3	13.1	1.84×10^{-8}
PAX6	paired box 6	9.7	7.5	4.5	0.0385	7.6	3.8	13.6	4.18×10^{-10}
NFIB	nuclear factor I/B	9.4	7.2	4.4	0.0582	9.3	5.7	12.8	1.18×10^{-8}
ID4	inhibitor of DNA binding 4	11.6	9.5	4.4	0.0724	11.8	8.5	10.1	2.79×10^{-8}
MYB	v-myb avian myeloblastosis viral oncogene homolog	6.4	4.3	4.3	0.0001	10.2	4.4	59	1.48×10^{-11}
NR4A3	nuclear receptor subfamily 4, member 3	6.3	4.3	4	0.0037	11.2	5.7	45.6	1.02×10^{-11}
REST	RE1-silencing transcription factor	9.1	7.2	3.8	0.0032	7.1	4.1	8	2.53×10^{-9}
KDM3A	lysine (K)-specific demethylase 3A	8.3	6.5	3.6	0.0262	8.9	5.8	8.9	1.86×10^{-9}
SOX2	SRY box 2	12.5	10.7	3.5	0.0301	12.1	9.2	7.3	3.92×10^{-8}
SOX9	SRY box 9	11.5	9.8	3.4	0.0255	9	4.4	24.2	2.16×10^{-10}
NR2F1	nuclear receptor subfamily 2, member 1	11.4	9.7	3.3	0.0165	11	7.8	8.9	1.46×10^{-9}
SALL1	spalt-like transcription factor 1	11.5	9.9	3.1	0.0278	9.9	4.6	38	2.76×10^{-10}
RFX3	regulatory factor X, 3	5.4	3.8	2.9	0.0031	9.2	5.2	16.5	1.01×10^{-9}

Top 15 Human and Mouse EZ Transport-related genes

Gene	Description	hEZ (log_2)	Peri hEZ (log_2)	Fold Change	p Value	mEZ (log_2)	Peri mEZ (log_2)	Fold Change	p Value
CFTR	cystic fibrosis transmembrane conductance regulator	9.5	5.1	21.2	3.86×10^{-6}	8.5	3.4	34.8	6.82×10^{-11}
SLC44A1	solute carrier family 44, member 1	9.5	7.3	4.5	0.0082	8.9	6.5	5.3	2.58×10^{-8}
OCA2	oculocutaneous albinism II	8.2	6.2	4.1	0.0026	6.2	4.0	4.6	6.24×10^{-6}
SLC15A2	solute carrier family 15, member 2	8.2	6.3	3.8	0.0069	7.5	4.7	7.1	0.0003
SLC38A6	solute carrier family 38, member 6	7.5	5.6	3.6	0.0842	7.0	5.5	3.0	5.09×10^{-5}
SLC40A1	solute carrier family 40, member 1	10.2	8.6	3.1	0.0473	7.2	5.8	2.7	0.0083
LRP6	low-density lipoprotein receptor-related protein 6	10	8.4	3	0.0099	7.7	6.4	2.4	0.0016
FOLR1	folate receptor 1 (adult)	6.6	5.1	2.9	0.0164	9.0	4.3	25.0	4.04×10^{-11}

(*Continued on next page*)

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001

Table 1. *Continued*

Top 15 Human and Mouse EZ Transport-related genes									
Gene	**Description**	**hEZ (log_2)**	**Peri hEZ (log_2)**	**Fold Change**	**p Value**	**mEZ (log_2)**	**Peri mEZ (log_2)**	**Fold Change**	**p Value**
SLC16A9	solute carrier family 16, member 9	8.7	7.2	2.9	0.006	8.1	5.6	5.4	8.88×10^{-7}
SLC22A4	solute carrier family 22, member 4	9.3	7.8	2.9	0.0151	7.0	4.0	7.7	3.30×10^{-7}
SLC35D1	solute carrier family 35 D1	8.5	7.1	2.6	0.0042	7.7	4.6	8.4	6.28×10^{-9}
SLC12A7	solute carrier family 12, member 7	8.6	7.4	2.4	0.0143	8.1	5.3	7.0	1.03×10^{-7}
SLC2A12	solute carrier family 2, member 12	6.8	5.6	2.4	0.0335	8.4	4.8	12.0	1.18×10^{-9}
SLC30A5	solute carrier family 30, member 5	5.8	4.6	2.3	0.0341	8.5	7.0	2.7	0.0018
ABCC1	ATP-binding cassette, member 1	9.3	8.2	2.2	0.0194	8.2	5.2	7.9	5.16×10^{-9}

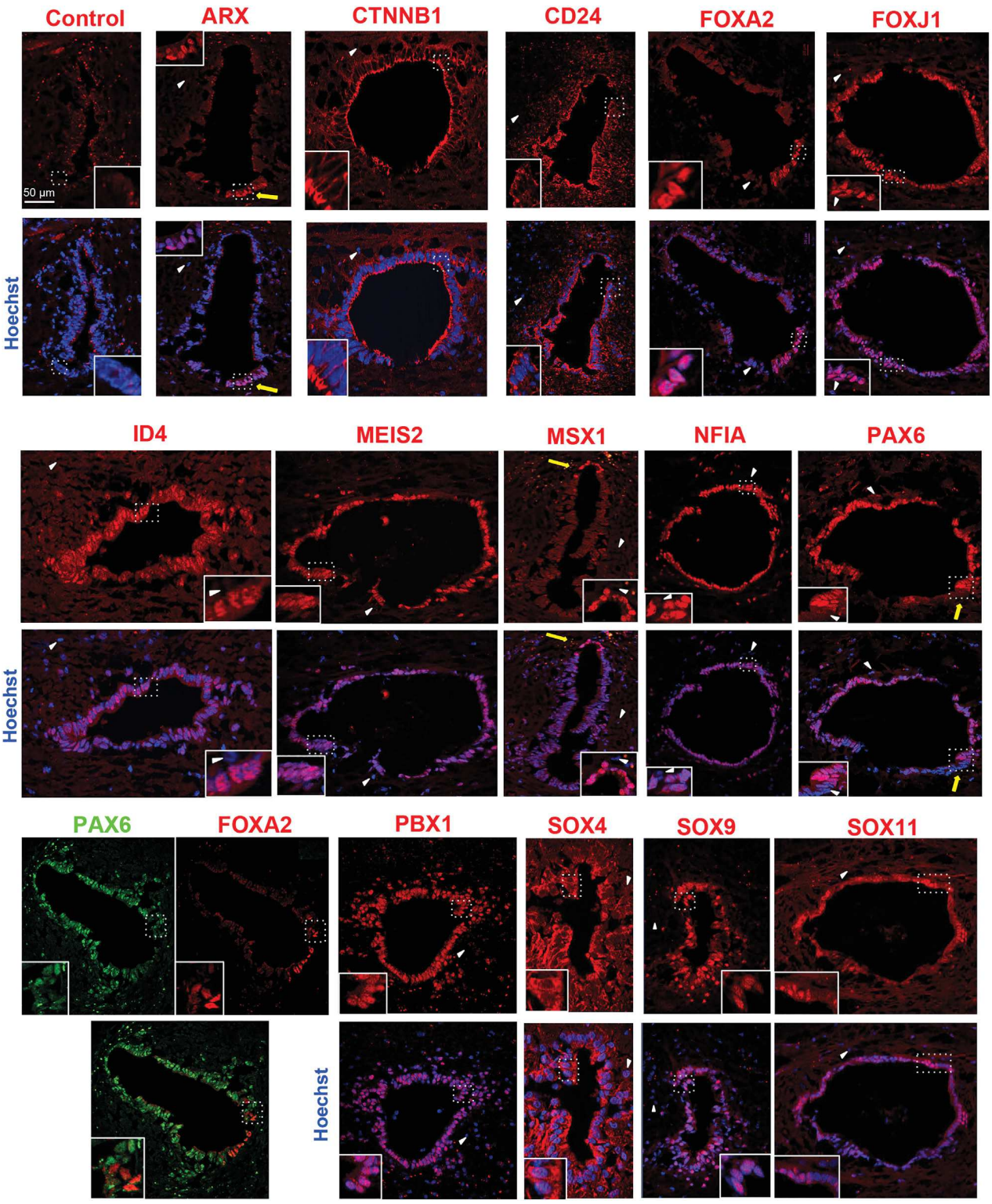
The table also shows lists of transporters and transcription factors (top 15) enriched in the mouse and human EZ. mEZ, mouse EZ; hEZ, human EZ.

(*Arx*, *Foxa1*, *Sulf1*) involved in SHH signaling, a pathway known to be crucial for ventral specification of spinal cord during development. The proteoglycan *Decorin* (*Dcn*) was also expressed in the ventral part, a gene positively regulated by SHH signaling (Ingram et al., 2008). To identify the cellular origin of *Dcn*, we performed double immunofluorescence (IF) for DCN and PKD2L1, a specific marker for CSF-N (Becker et al., 2018) (Figure S2). Indeed, co-localization indicated that these neurons express a high level of DCN, a result supported by recent single cell RNA-sequencing (RNA-seq) databases (Zeisel et al., 2018) (Figure S5A). Contrasting with ventral SHH signaling, a robust expression of two bone morphogenetic protein (BMP) morphogens (namely *Bmp6* and *Gdf10/Bmp3b*) involved in the dorsal patterning of the developing spinal cord (Wilson and Maden, 2005) were detected in the dorsal EZ (Figure 1G and Table S5). Two genes, *Prokr2* and *Thbs2*, modulating neural stem cell fate in the SVZ (Benner et al., 2013; Prosser et al., 2007), were also enriched in the dorsal part. The dorsal or ventral expression of *Arx*, *Bmp6*, *Dcn*, *Foxa1*, *Gdf10*, *Prokr2*, *Sulf1*, and *Thbs2* in the EZ was confirmed in the Allen brain atlas (Figure S3A).

The Mouse and Human EZ Show a Conserved Dorsal-Ventral Regionalization of Transcription Factor Expression

One hundred twenty-one TFs were found more specifically-expressed (fold change EZ/periEZ $\geq$ 2) in the human and mouse EZ (Figure 1E). Examples of expression in the Allen brain atlas are illustrated in Figure S1B. IF confirmed the presence of corresponding proteins for some of them (ARX, FOXJ1, ID4, MEIS2, MSX1, NFIA, PAX6, PBX1, SOX4, SOX9, and SOX11) both in human (17-year-old) and mice (Figures 2 and 3). The histological quality of the second human spinal cord (46-year-old) was reduced compared with that of the first patient, although we could confirm protein expression for FOXJ1, ID4, MEIS2, NFIA, and PAX6 in this sample (Figure S4A). Unexpectedly, some of these TFs showed a dorsal-ventral asymmetric expression. Both in human (17-year-old) and in mouse, PAX6 protein stained dorsal and lateral cells while ventral cells were negative (Figures 2 and 3), which was also observed in the GENSAT gene expression atlas (Figure S1B). Another clear regionalized-expression was found for MSX1 protein in mouse and in human (17-year-old). In the human sample, MSX1 was weakly expressed by ependymal cells but cells in the dorsal part had a much stronger staining (Figure 2). In mice, MSX1 protein was confined to a few cells constituting the roof of the EZ (Figure 3), which was confirmed in gene expression atlases (Figure S3B). These MSX1$^+$ cells typically exhibit a higher level of ID4 staining in mice (Figure 3). Contrasting with

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001



(legend on next page)

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001

the dorsally-expressed proteins, we detected a ventral expression of ARX, a TF expressed by the floor-plate during spinal cord development. ARX protein was confined to a group of ventral cells both in human and mouse EZ (Figures 2 and 3). This led us to investigate by IF the expression of FOXA2, another TF involved in floor plate formation (Cho et al., 2014). Foxa2 specificity for the EZ was under our selected threshold in mice and in human (fold change EZ/periEZ = 1.7 and 1.3, respectively) however the FOXA2 protein was detected in a group of ventral and ventral/ventro-lateral cells in human and mice respectively (Figures 2 and 3). In human, the same population of ventral cells expressed ARX and FOXA2 (Figure S4B) whereas in mice, FOXA2 was expressed by ventral CSF-N expressing DCN and PKD2L1 but did not express ARX (Figure S2). In addition to these evolutionarily-conserved TFs, we also detected genes whose expression appears to be specifically expressed in the mouse or the human EZ (Table S3). This was checked at the protein level for *Tal1* (also known as *Scl*), a well-known TF involved in hematopoiesis but also expressed during spinal cord development (Smith et al., 2002). IF for TAL1 revealed the presence of positive subpopulations in the mouse EZ (Figure S2) which co-labeled with PDK2L1, thus identifying these cells as CSF-N. This was confirmed by single cell CNS RNA-seq database (Zeisel et al., 2018) (Figure S5A). In contrast, no convincing staining for TAL1 and PDK2L1 proteins was detected in the two human spinal cord EZs (not shown), and DCN labeling was restricted to vessels in the parenchyma (Figure S5B) as expected from the literature (Jarvelainen et al., 2015). A recent single cell CNS RNA-seq database (Zeisel et al., 2018) identified six markers for mouse CSF-N (*Crct1*, *Dcn*, *Espn*, *Pkd1l2*, *Pkd2l1*, and *Pdzk1ip1*), which we found enriched at the transcriptional level in the mouse EZ but not in the human EZ (Figure S5C).

Collectively, these results indicate a conserved dorsal-ventral regionalization of TF expression in the mouse and human EZ; however, no evidence could be found for the presence of CSF-N in human.

Vegfr3-YFP Transgenic Mice Reveal Distinct MSX1+ and ARX+ Cells in the Roof and Floor of EZ

The regionalized expression of TFs in the EZ was indicative of specific cells located in the roof and floor of the EZ. This was also suggested by the specific expression of the immature neural marker NES in these regions (Becker et al., 2018). The Vegfr3-YFP transgenic mouse has been used to detect subpopulations of neural precursor cells in the neurogenic SVZ (Calvo et al., 2011), which prompted us to use it in the spinal cord EZ context. Indeed, subpopulations of EZ cells expressing YFP were observed in all sections and were specifically enriched at the roof and floor portions (Figures 4A and 4B). These cells were positive for TFs strongly expressed by ependymal cells such as FOXJ1 and SOX2 while only the dorsal YFP+ cells express PAX6 (Figure 4C). In addition, in the roof part, 67% (41 among 61 cells, two mice) of YFP+ cells expressed the MSX1 TF (Figure 4C), which was further confirmed by generating a double transgenic mouse (Vegfr3-YFP × Msx1-Tomato, see below, Figure 6F) in which approximately 77% of the dorsal YFP+ cells were also Tomato+ (37 among 48 cells, two mice). It was previously reported that the c-RET receptor is present in a subpopulation of radial dorsal cells in the EZ (Pfenninger et al., 2011), and we observed that 71% of YFP+ (20 among 28 cells, two mice) were positive for this receptor (Figure 4C). In the EZ floor, 90% of YFP+ cells (60 among 66 cells, three mice) expressed the ARX TF (Figure 4C). Finally, some dorsal Vegfr3-YFP+ cells were clearly positive for GFAP and NES intermediate filaments (Figure S6A), whereas we could not establish this with confidence for ventral cells. These results support the existence of distinct EZ roof and floor cells expressing *Vegfr3* and specific TFs.

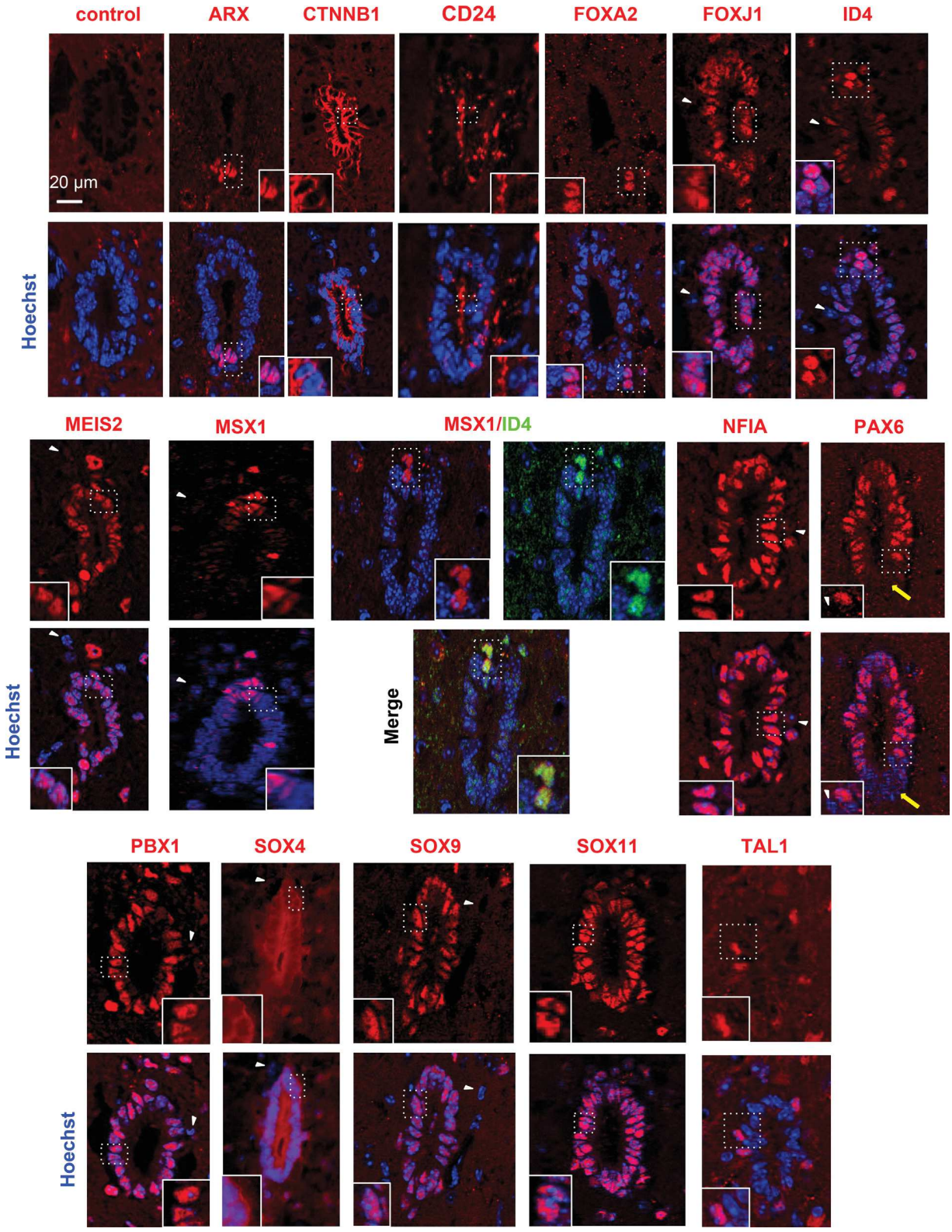
Roof MSX1+ and Floor ARX+ Ependymal Cells are Born Early during Development

We next questioned the developmental origin of MSX1+ and ARX+ cells by performing IF at multiple developmental stages (embryonic day 13 [E13], E18, postnatal day 1 [P1], P6, P22, and P38) (Figure 5A). At E13, a group of MSX1+ cells was detected at the dorsalmost part of the developing spinal cord while a group of ARX+ cells were present in the ventralmost region. These cells, situated at positions corresponding respectively to the embryonic floor and roof plates, had elongated nuclei oriented along the dorsal-ventral axis, evocating migration. At E18, ARX+ and MSX1+ cells appeared to have migrated centrally to delimit a small group of cells presumptive of the adult EZ. At P1 and P6, the number of ARX+ and MSX1+ cells was reduced and at P22 and P38 only a few cells remained in the roof and floor of the EZ (Figure 5A). To analyze

Figure 2. Human EZ Characterization

IF of the indicated proteins in the human EZ (patient aged 17 years). White arrowheads show negative cells indicative of the staining specificity. Yellow arrows show ventral ARX+ cells, dorsal MSX1+ cells, and ventral PAX6− cells. Note that SOX4 is mostly cytoplasmic. Images are oriented with ventral part at bottom. Labelings were performed at the thoracic (control, ARX, CD24, FOXA2, MSX1, SOX4, SOX9) or lumbar level (CTNNB1, FOXJ1, ID4, MEIS2, NFIA, PAX6, PBX1, SOX11). These images are representative of at least eight sections. Scale bar, 50 μm (applies to all images).

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001



(legend on next page)

further the developmental origin of adult EZ MSX1$^+$ cells, we used a genetic tracing approach based on a Msx1-CreERT2/Rosa-Loxed Tomato transgenic line (hereafter referred to as Msx1-Tomato [Lallemand et al., 2013]). Tamoxifen injection in adult mice revealed the presence of Tomato$^+$ cells residing in the EZ roof (Figures 6A and S6B), which validated this tool. Tomato$^+$ cells were very rarely observed in the ventral part of the EZ or in the parenchyma (not shown). Tamoxifen was then injected in pregnant transgenic mice at E11.5 to permanently label embryonic MSX1$^+$ cells and explore their fate (Figure 5B). Analysis of sections taken from cervical and lumbar spinal cord levels of P30 mice (n = 2 mice) revealed the constant presence of Tomato$^+$ cells situated in the EZ roof (Figure 5B). Altogether, these data demonstrate an early embryonic origin of ARX$^+$ and MSX1$^+$ spinal cord EZ cells.

Roof MSX1$^+$ Cells Are Radial Quiescent Cells

We previously reported an enrichment of neurosphere-forming cells in the dorsal half of the EZ (Sabourin et al., 2009). This was associated with a high content of GFAP$^+$ radial glial-like cells. As MSX1$^+$ cells were found almost exclusively in the roof of the EZ, further characterization of these cells was done. This was also motivated by the reported expression of *Msx1/2* in some stem cells during eye development (Bélanger et al., 2017) and its role in promoting regeneration of amputated tail including spinal cord (Beck et al., 2003) in *Xenopus*. The intense fluorescence observed in Msx1-Tomato mice enabled morphological characterization of these cells, notably using clarification of the whole spinal cord and 3D reconstruction. Tomato$^+$ cells have a long radial morphology and send their process toward the pial surface (Figures 6A, 6B, and S6B). Their soma make contact with the lumen, but radial Tomato$^+$ cells can also be observed more dorsally and at distance from the EZ (Figure 6A, yellow arrow). Horizontal sections also revealed the existence of Tomato$^+$ processes running longitudinally along the EZ roof (Figure 6C).

By performing IF for c-RET, FOXJ1, GFAP, NES, and SOX2, we observed that most Tomato$^+$ cells also express these markers (Figures 6D and S6C). We next crossed Msx1-Tomato mice with hGFAP-GFP transgenic mice, which have been widely used to purify adult neural stem cells (Nolte et al., 2001). Contrasting with GFAP immunostaining (Figure S6C), only a small fraction of Tomato$^+$ cells (15%, 8 double-positive cells among 54 Tomato$^+$ cells; n = 2 mice) were GFP$^+$ (Figure 6E). Often GFP$^+$ and Tomato$^+$ cells were found to be associated, suggesting close interactions between them. We then explored the proliferation rate of Tomato$^+$ cells by injecting tamoxifen for 5 days in Msx1-Tomato mice to label MSX1$^+$ cells, then 5-ethynyl-2′-deoxyuridine (EdU) injections were performed for 5 additional days (twice a day) before sacrificing on day 11 (Figure 6G). As previously reported, few EdU$^+$ cells were found in the EZ (approximately one cell per section), however no Tomato$^+$ EdU$^+$ could be observed (Figure 6G). This indicates that MSX1$^+$ cells are quiescent or proliferate at a much slower rate compared with the other ependymal cells.

DISCUSSION

In this article, RNA profiling was used to generate a cellular and molecular resource for the adult human and mouse spinal cord EZ. Our findings reveal important and new characteristics of this poorly defined region.

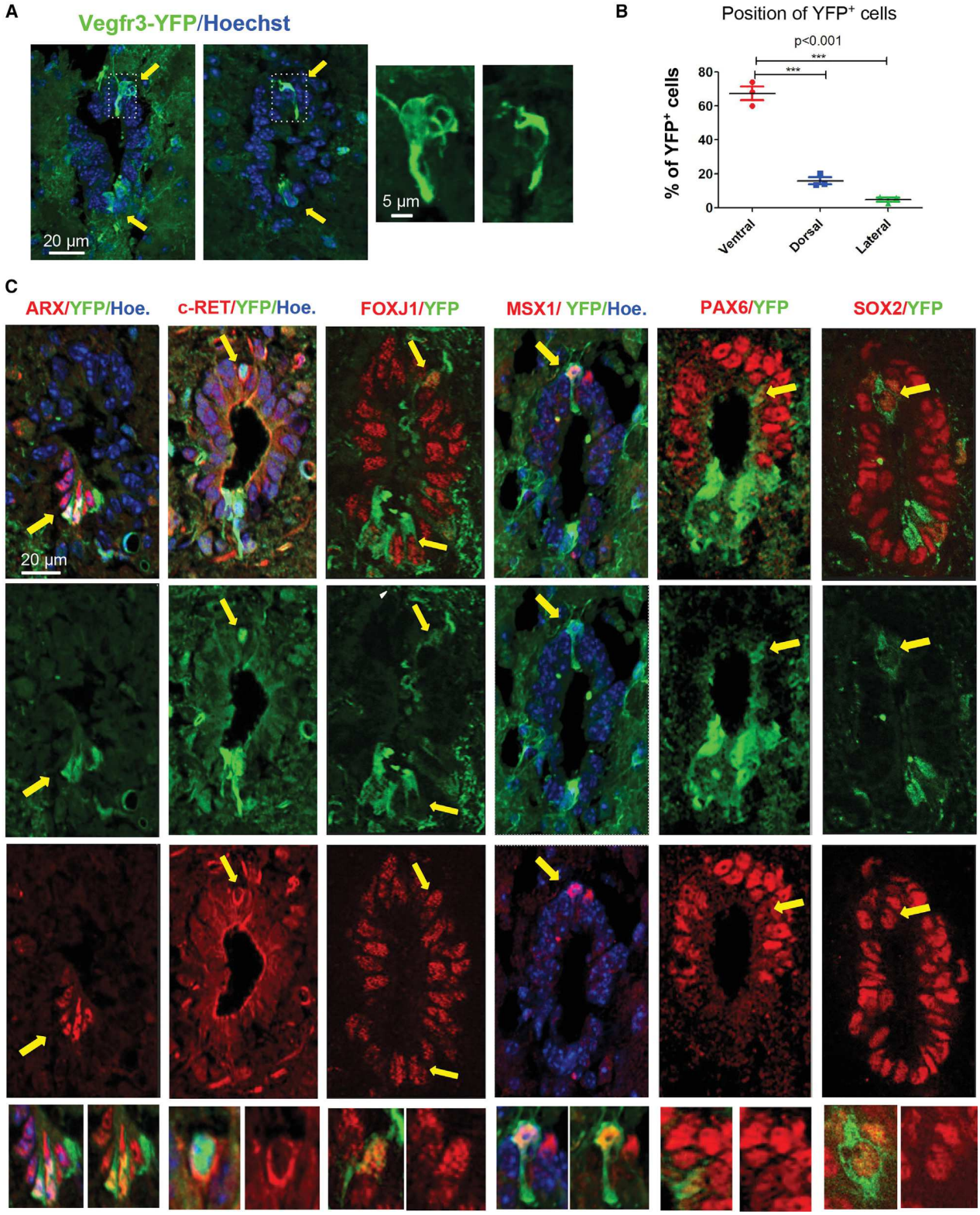
In accordance with the presence of neural stem cells in the EZ, this region shows enrichment for smoothened/SHH and Hippo/YAP signaling genes, which are involved in stem cell maintenance (Alvarez-Buylla and Ihrie, 2014; Mo et al., 2014). Echoing their localization at the interface between CSF and the spinal cord parenchyma, ependymal cells also highly express genes involved in transport and ciliogenesis including three TFs of the RFX family and FOXJ1 (Figure 1E). Compared with brain, spinal cord ependymal cells proliferate (Alfaro-Cervello et al., 2012; Pfenninger et al., 2011), which is also reflected at the RNA level by enrichment for genes involved in cell division (Figure 1E; Tables S1 and S2). A set of 120 TFs conserved between mice and humans was identified and the expression at the protein level was confirmed for 12 of them. MEIS2 and PBX1 expressions are particularly interesting, as these TFs dimerize and are essential regulators of adult SVZ neurogenesis (Grebbin et al., 2016). Ependymal cells also express high levels of NFIA and SOX9, two TFs involved in gliogenesis during development (Kang et al., 2012). Post lesion, these cells mostly generate astrocytes (Barnabé-Heider et al., 2010) and the expression of NFIA and SOX9 may be responsible for their glial fate restriction.

One unexpected observation was the conserved and regional expression of four homeodomain-containing TFs

Figure 3. Mouse EZ Characterization

IF for the indicated proteins in the adult mouse EZ (lumbar level). Images are oriented with ventral part at bottom. The yellow arrows on PAX6 staining show negative cells in the ventral part. Note the higher expression of ID4 in cells localized in the dorsal part, which also express MSX1. FOXA2$^+$ and TAL1$^+$ cells are localized in a subependymal position and express PKD2L1 a marker specific for CSF-N (Figure S2). Note that SOX4 is mostly cytoplasmic. These images are representative of ten sections per animal, n = 4 mice analyzed. Scale bar, 20 μm (applies to all images).

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001



(legend on next page)

in the niche, namely ARX, FOXA2, MSX1, and PAX6. This situation is reminiscent of the developing spinal cord neuroepithelium where cells along the dorsal-ventral axis express different combinations of homeogenes (Wilson and Maden, 2005). Both in human and mouse, ARX and FOXA2 are expressed by ventralmost cells that do not co-express PAX6. Of note, whereas FOXA2 was expressed by ventral CSF-N in mice (Figure S2 and Petracca et al., 2016), no such cells have been identified in human so far. The two other markers we identified in mice for these cells, DCN, an extracellular matrix protein (Jarvelainen et al., 2015), and TAL1, a hematopoietic transcription factor, were not observed in the human EZ. This indicates a divergent organization of the ventral EZ between rodents and humans. In the dorsal portion of the EZ, high MSX1 expression was restricted to few cells located in the EZ roof both in mouse and human. By tracing the origin of ARX$^+$ and MSX1$^+$ cells during spinal cord development in mice, we observed that they are already present at E13.5 in the spinal cord roof and floor plates. Their number is then reduced and they appear to migrate centrally to generate the roof and floor of the adult EZ. The early developmental origin (at least E11.5) of MSX1$^+$ cells was further demonstrated using genetic tracing. Thus, in contradiction to what was previously established (Fu et al., 2003), ependymal cells are not entirely derived from the ventral neuroepithelium but also incorporate cells from the dorsal part of the developing spinal cord. Another distinguishing feature of the dorsal and ventral EZ cells is the expression of *Vegfr3* (also known as *Flt4*, the receptor for VEGFC), which was observed using Vegfr3-YFP transgenic mice. Part of these Vegfr3-YFP$^+$ cells express MSX1 and ARX. During brain development *Vegfr3* is expressed in the ventricular zone and radial glial cells (Ward and Cunningham, 2015). It is also expressed in brain adult neural stem cells where it regulates their activation and proliferation (Calvo et al., 2011). A similar role for *Vegfr3* may apply for adult spinal cord ependymal cells.

These results provide evidence for the persistence of embryonic floor and roof plate cells in the adult spinal cord niche. Recent studies, based on a Wnt-reporter mouse labeling dorsal neural tube cells (Xing et al., 2018) and on a Nato3-reporter mouse labeling floor plate cells (Khazanov et al., 2017), reached similar conclusions. During development, these floor and roof plate cells secrete morphogens such as SHH and BMP6, acting as growth factors and patterning signals (Wilson and Maden, 2005). Our results (Figure 1G) and expression atlas (Figure S3A) indicate that dorsal EZ cells express morphogen genes (*Bmp6* and *Gdf10*). This suggests a specific role for these cells in the spinal cord niche, which warrants further exploration.

As the presence of MSX1$^+$ cells has not been reported in the spinal cord EZ, we characterized these cells further and observed that they have distinct features compared with the other cells of the niche. They have a long radial morphology and express FOXJ1, GFAP, NES, and SOX2 proteins. They also express c-RET, the receptor for GDNF and NTN growth factors, which is important for hematopoietic stem cells and neural crest cells (Kubota et al., 2004). By crossing Msx1-CreERT2/Tomato and hGFAP-GFP transgenic mice, only a minority of Tomato$^+$ cells were double-positive, thus revealing the presence of a cellular heterogeneity in the dorsal part of the niche and of MSX1$^+$ cells.

The existence of subpopulations of quiescent neural stem cells has been reported in the hippocampus and the SVZ niches (Codega et al., 2014; Llorens-Bobadilla and Martin-Villalba, 2017; Llorens-Bobadilla et al., 2015). Importantly, data mining of single cell RNA-seq analysis performed in the SVZ revealed that *Msx1* and *Id4* are highly enriched in quiescent neural stem cells (Llorens-Bobadilla et al., 2015) (Figure S5D). These two TFs are regulated by the BMP signaling (Ramos and Robert, 2005; Ruzinova and Benezra, 2003) and, indeed, expression of *Bmp6* and *Bmp3b/Gdf10* is also restricted to SVZ quiescent stem cells (Figure S5D). A similar situation may also be present in the dorsal part of the spinal cord EZ. Indeed, we found that *Bmp6*, *Bmp3b/Gdf10*, and *Msx1* are highly expressed in the dorsal part of the spinal cord EZ and that MSX1$^+$ cells express a higher level of ID4 transcription factor (Figure 3). EdU incorporation also revealed that MSX1$^+$ cells are less proliferative than the other cells of the niche (Figure 6G). This suggests that dorsal radial MSX1$^+$ cells may behave as quiescent neural stem cells in the adult spinal cord. Further work is needed to support this hypothesis.

Figure 4. EZ in Vegfr3-YFP Mice

(A) Representative images of the spinal cord EZ (lumbar level) in Vegfr3-YFP mice (IF anti-GFP). Images are oriented with ventral part at bottom. YFP$^+$ cells are mainly present in the dorsal and ventral regions (yellow arrows). Boxed areas show dorsal cells sending a process toward the lumen. These images are representative of at least 20 sections per animal (n = 3 mice analyzed). Ventral YFP$^+$ cells are present in >95% of sections whereas dorsal YFP$^+$ cells are present in approximately 25% of examined sections.

(B) Quantification of YFP$^+$ cells in the lumbar EZ (187 YFP$^+$ cells counted) indicated a preferential ventral and dorsal localization. One-way ANOVA + Tukey's post test (n = 3 mice).

(C) Phenotypic characterization of YFP$^+$ cells with indicated protein (images are representative of 20 sections, n = 3 mice). Yellow arrows show double-positive cells. Images at the bottom are high magnification of arrow-pointed areas. Hoe., Hoechst. Scale bar, 20 μm (applies to all images).

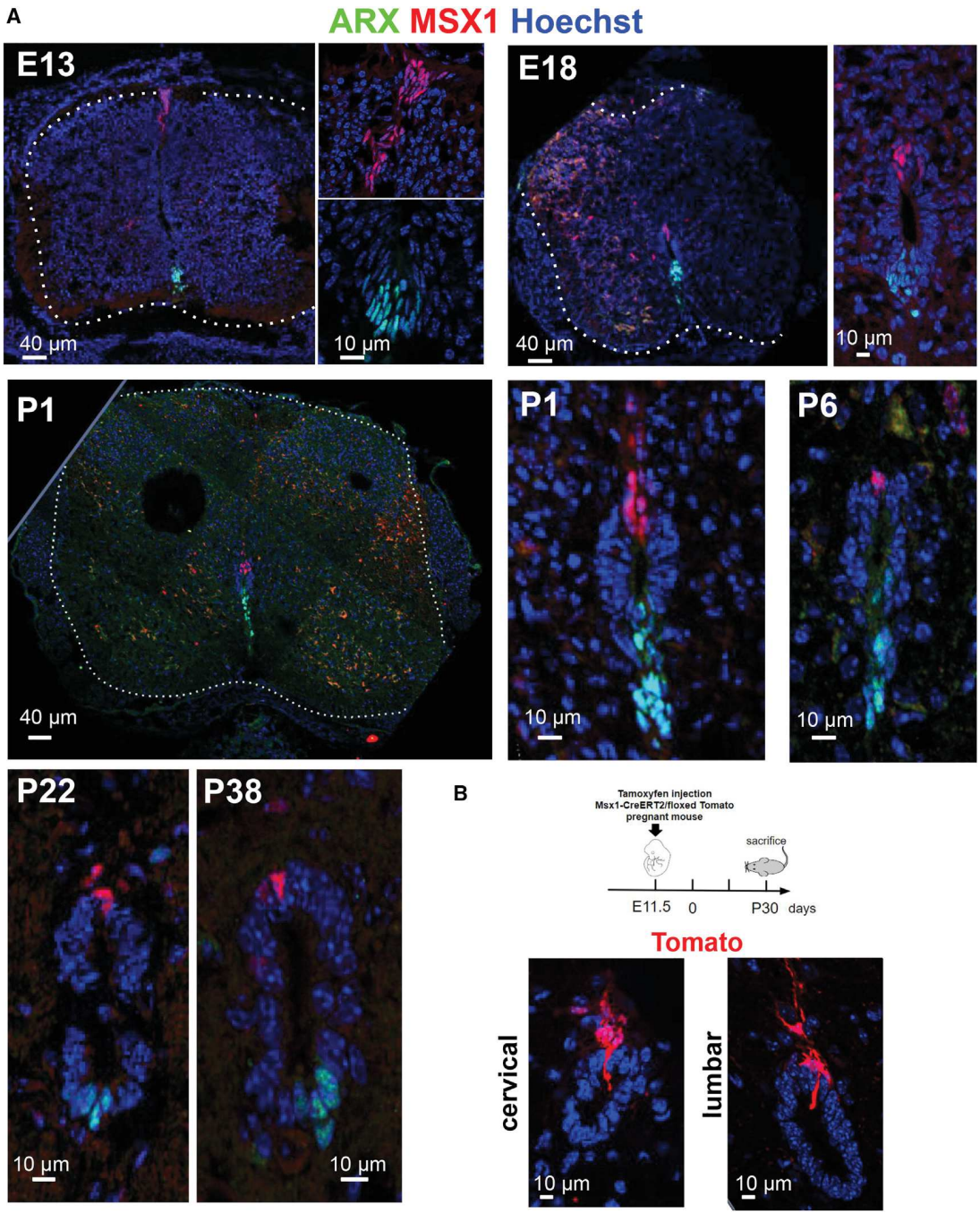


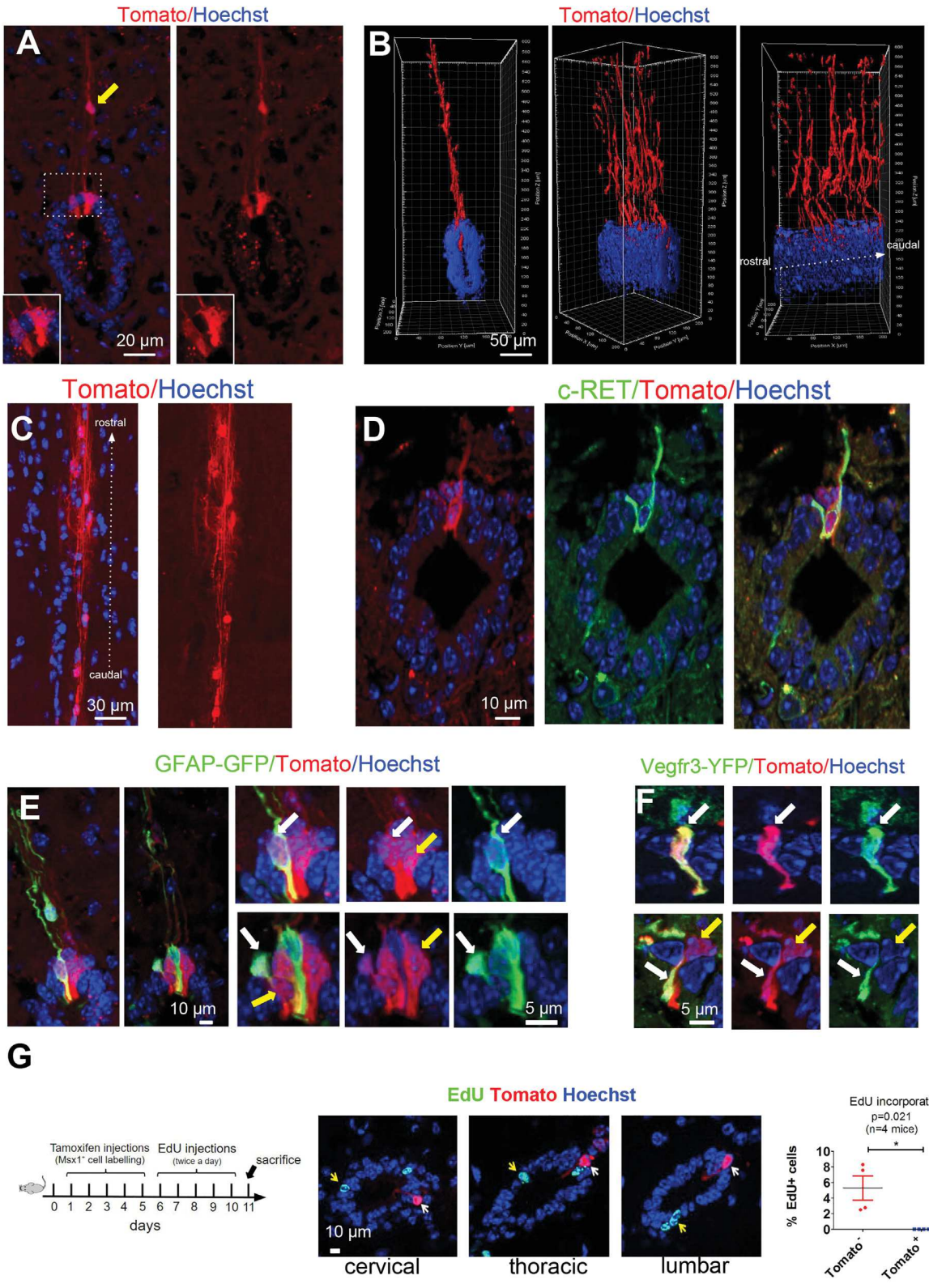
Figure 5. MSX1 and ARX Expression in the Developing Spinal Cord

(A) IF for MSX1 and ARX during spinal cord embryonic development (E13 and E18) and postnatal stages (P1, P6, P22, P38). All images are oriented with ventral part at bottom. Images are representative of ten sections (n = 2 embryos and pups analyzed per stage).

(B) Representative images (n = 20 sections each levels, two mice) of the EZ of a P30 Msx1-CreERT2/Rosa-Tomato mouse derived from an embryo subjected to tamoxifen at E11.5.

In summary, our results uncovered that the adult spinal cord EZ region is conserved, regionalized, and composed of a mosaic of cells with different embryonic origin and expressing different types of TFs. This corpus of knowledge on the organization and genes expressed in the EZ will help to explore this adult stem cell niche further and will also be

Please cite this article in press as: Ghazale et al., RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1+ Roof-Plate-Derived Cells, Stem Cell Reports (2019), https://doi.org/10.1016/j.stemcr.2019.04.001



(legend on next page)

useful to shed light on ependymoma, a rare type of tumor that can arise in the human spinal cord EZ.

EXPERIMENTAL PROCEDURES

Human Samples

Human spinal cords were collected at the Montpellier Hospital from two organ-donor patients (17 [male] and 46 [female] years old, accidental death) in strict agreement with the French bioethics laws (articles L1232-1 and -6) and after approval by the French institution for organ transplantation. An informed consent from the families was obtained by the organ procurement organization for this study. Surgery was performed as described previously (Bauchet et al., 2013), and the thoracolumbar segments were immediately placed in liquid nitrogen before processing for microdissection and immunofluorescence.

Animals

Mice were handled following the guidelines of the Animal Care and Use Committee of the National Institute of Health and Medical Research (INSERM) who approved this study in accordance with the European Council directive (2010/63/UE) for the protection and use of vertebrate animals. Adult CD1 mice (3 months, Charles River, France) were used for microdissection, RNA profiling, and histology. Msx1-CreERT2/Rosa-Loxed Tomato transgenic line (Lallemand et al., 2013) was obtained from Y. Lallemand (Pasteur Institute, Paris). To induce recombination in Msx1-CreERT2 animals, we injected 100 μL of tamoxifen (Sigma, T5648, 20 mg/mL dissolved in corn oil) intraperitoneally for 4–5 days. hGFAP-GFP (Nolte et al., 2001) and Vegfr3-YFP (Calvo et al., 2011) transgenic mice were obtained from Prof. H. Kettenmann (MDC, Berlin) and Dr. J.L. Thomas (ICM, Paris), respectively.

Tissue Microdissection

For human and mouse, after collection the spinal cords were flash frozen in N_2 without chemical fixation. Frozen sections (30 μm thick, T9-T10 thoracic part for mouse and thoracolumbar for human) were obtained at −23°C to prevent RNA degradation using a CM3050S microtome (Leica Microsystems, Wetzlar, Germany) and were mounted on PEN-membrane 1-mm glass slides (P.A.L.M. Microlaser Technologies, Bernried, Germany) that had been pretreated to inactivate RNase. Sections were then fixed in a series of pre-cooled ethanol baths (40 s in 95%, 75% and 30 s in 50%), stained with cresyl violet 1% for 30 s, and dehydrated in a series of pre-cooled ethanol baths (30 s in 50%, 75%, and 40 s in 95% and 100%). Immediately after dehydration laser microdissection was performed using a PALM MicroBeam microdissection system version 4.6 equipped with PALM RoboSoftware (P.A.L.M. Microlaser Technologies). Laser power and duration were adjusted to optimize capture efficiency, and microdissection was performed at 63× magnification. Samples were collected in adhesive caps (P.A.L.M. Microlaser Technologies). To limit RNA degradation, we collected samples for up to 15 min per slide and lysed microdissected tissue with 250 μL of lysis buffer (Promega, Madison, WI, USA). The samples were stored at −80°C until extraction was performed using the ReliaPrep RNA cell Miniprep System (Promega) according to the manufacturer's protocol and eluted with 14 μL of RNase-free water. The concentration of RNA was determined using Nanodrop 1000 and the integrity of RNA was determined using the RNA 6000 Pico Kit and Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number was above 7/8.

RNA Profiling and Bioinformatics Analysis

RNA profiling was performed using Affymetrix microarray technology. Hybridization targets were obtained following a double-amplification procedure according to the protocol developed by Affymetrix (GeneChipTwo-Cycle Eukaryotic Target Labeling Assay; Affymetrix, USA). A hybridization mixture containing 10 μg of biotinylated cRNA was generated. The biotinylated cRNA was hybridized to HT_HG-U133_Plus_PM (human) and HT_MG-430_PM (mouse) Affymetrix microarrays. Four mouse and two human samples (EZ and peri EZ regions each, Figure 1A) were analyzed in total. The microarrays were scanned using the Affymetrix Gene Atlas scanner. The data files were generated with Affymetrix Expression Console v1.2.1 and gene expression data were normalized with the GC-RMA algorithm. Gene expression profiles were analyzed using the Affymetrix TAC 4.0 software (Transcriptome Analysis Console). The filter criteria were set to a linear fold change ≥ 2 between EZ and peri EZ regions. Gene lists were analyzed with DAVID Bioinformatics Resources 6.83 for gene enrichment analysis (Huang et al., 2008).

Human and Mouse Histology

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused intracardially with 10 mL of PBS followed by 50 mL of 4% formaldehyde-PBS solution (pH 7.0). After dissection, spinal cords were post-fixed in the same solution for 1 h at 4°C and cryopreserved by successive immersion in 10%, 20%, and 30% sucrose solutions in PBS for at least 6 h. Cervical, thoracic and lumbar parts of the spinal cord were cut,

Figure 6. Characterization of Dorsal MSX1+ Cells

(A) Radial morphology of MSX1+ cells observed in Msx1-CreERT2/Rosa-Tomato mice. Yellow arrow shows rare radial Tomato+ cells outside EZ. Images are oriented with ventral part at bottom.

(B) 3D reconstruction of Tomato+ cells (coronal, intermediate, and lateral views).

(C) Dorsal view of the dorsal EZ region showing rostral-caudal oriented Tomato+ processes.

(D) Expression of c-RET receptor in Tomato+ cells (n = 10 sections).

(E and F) Representative images (n = 20 sections, two mice) of the EZ in double transgenic hGFAP-GFP/Msx1-CreERT2/Rosa-Tomato (E) and Vegfr3-YFP/Msx1-CreERT2/Rosa-Tomato (F) mice. White arrows indicate double-positive cells and yellow arrows Tomato+-only cells.

(G) EdU incorporation (5 days) in the EZ. Images show EdU+ cells (yellow arrows) not positive for Tomato (white arrows) in Msx1-CreERT2/Rosa-Tomato mouse sections. Quantifications are provided on right-hand graph (112 spinal cord sections, 5,120 cells examined, unpaired t test, n = 4 mice).

embedded in OCT medium, rapidly frozen in liquid N_2-cooled isopentane, and cryosectioned (14 μm) (Leica apparatus). For developmental studies using time-mated embryos (Figure 5A), the day the plug was found was considered as E0.5. The embryos and pups (entire animal for P1, P6, and dissected spinal cord for P22) were fixed by direct immersion in 4% formaldehyde-PBS solution for 1 h then processed as for the adult spinal cord. P38 animals were perfused intracardially and post-fixed as for adults. To access the proliferation rate of Tomato+ cells (Figure 6G), we injected adult mice with EdU twice a day for 5 days (50 mg/kg), perfused them intracardially, and processed them for EdU staining (Baseclick kit).

For human, unfixed spinal cords at thoraco or thoracolumbar levels were directly cryosectioned (20 μm) and sections were immediately fixed by direct immersion in ice-cooled 4% formaldehyde-PBS solution for 20 min, followed by three washes with PBS.

Immunofluorescences were performed with primary antibodies (listed in Table S6) on sections permeabilized for 1 h with 0.1% Triton X-100 and 5% donkey serum. Secondary antibodies (Alexa 488- or Alexa 594-conjugated species-specific anti-mouse, -rabbit, or -goat) were purchased from The Jackson Laboratories. Incubations without primary antibody or with antibody recognizing antigens not present in the sections (monoclonal anti-DYKDDDDK tag or polyclonal antibodies against GFP) were used as negative controls. Nuclei (blue in all images) were stained with 1 μg/mL Hoechst for 10 min. The quality of staining was evaluated by two independent investigators (J.-P.H. and H.G. or C.R.). Images were taken using optical sectioning with structured illumination (Zeiss apotome microscope) or using a multi-photonic microscope (Zeiss LSM 7MP OPO) (Figure 6B). All presented images for mice are representative images, and the number of examined sections and animals are indicated in the figure legends. For human labelings, images are representative of at least eight sections for the first (17-year-old) patient and four sections for the second (46-year-old) patient. Clarification of mouse spinal cord was performed with protocol described in Tomer et al. (2014), and Imaris software was used for image treatments and 3D reconstruction.

Statistical Analysis

All experiments and stainings were performed at least twice, most of them three times. Data are represented as means ± standard error of mean. Statistical differences in experiments were analyzed with tests indicated in the figure legends (GraphPad Prism software). Significance is denoted by ***$p < 0.001$, **$p < 0.01$, and *$p \leq 0.05$.

ACCESSION NUMBERS

Data are available at the functional genomics data Gene Expression Omnibus (GEO: GSE118445).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.04.001.

AUTHOR CONTRIBUTIONS

C.R., H.G., N.L., D.M., and J.-P.H. performed and analyzed most of the experiments. J.-P.H. wrote the article. F.E.P., H.N.N., S.A., S.M., L.J., and B.R. contributed to spinal cord histology. Y.G. performed clarification and 3D reconstruction of spinal cord. L.B. performed surgery for human spinal cord and V.R. provided human spinal cord tissues. C.-F.C, J.-L.T., Y.L., and E.H. provided reagents and mouse models as well as intellectual input.

ACKNOWLEDGMENTS

This work was supported by grants from IRP (Switzerland), IRME (France), AFM (France), ANR EU ERANET Neuroniche (J.-P.H.), and ANR Brainwash (J.-L.T., L.J.). H.G. was supported by an AFM PhD fellowship. We thank all Montpellier biocampus facilities for help (RHEM, MRI RIO, RAM) and excellent technical work. We are very grateful to Dr. H Boukhaddaoui (imaging), Dr. C Duperray (cytometry), M. Maistre (laser microdissection facilities, Bordeaux), V. Pantesco (Affymetrix facilities), and M. Goussard/P. Guigue (animals) for providing technical expertise in this work. We warmly thank Prof. Morohashi (Kyushu University, Japan) for anti-Arx antibody. The authors declare that there is no conflict of interest regarding the publication of this article.

Received: September 4, 2018
Revised: March 29, 2019
Accepted: April 1, 2019
Published: April 25, 2019

REFERENCES

Adrian, E.K., Jr., and Walker, B.E. (1962). Incorporation of thymidine-H3 by cells in normal and injured mouse spinal cord. J. Neuropathol. Exp. Neurol. *21*, 597–609.

Alfaro-Cervello, C., Soriano-Navarro, M., Mirzadeh, Z., Alvarez-Buylla, A., and Garcia-Verdugo, J.M. (2012). Biciliated ependymal cell proliferation contributes to spinal cord growth. J. Comp. Neurol. *520*, 3528–3552.

Alvarez-Buylla, A., and Ihrie, R.A. (2014). Sonic hedgehog signaling in the postnatal brain. Semin. Cell Dev. Biol. *33*, 105–111.

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

Bauchet, L., Lonjon, N., Vachiery-Lahaye, F., Boularan, A., Privat, A., and Hugnot, J.-P. (2013). Isolation and culture of precursor cells from the adult human spinal cord. Methods Mol. Biol. *1059*, 87–93.

Beck, C.W., Christen, B., and Slack, J.M. (2003). Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. Dev. Cell *5*, 429–439.

Becker, C.G., Becker, T., and Hugnot, J.-P. (2018). The spinal ependymal zone as a source of endogenous repair cells across vertebrates. Prog. Neurobiol. *170*, 67–80.

Bélanger, M.-C., Robert, B., and Cayouette, M. (2017). Msx1-positive progenitors in the retinal ciliary margin give rise to both neural and non-neural progenies in mammals. Dev. Cell *40*, 137–150.

Benner, E.J., Luciano, D., Jo, R., Abdi, K., Paez-Gonzalez, P., Sheng, H., Warner, D.S., Liu, C., Eroglu, C., and Kuo, C.T. (2013).

Protective astrogenesis from the SVZ niche after injury is controlled by Notch modulator Thbs4. Nature *497*, 369.

Calvo, C.-F., Fontaine, R.H., Soueid, J., Tammela, T., Makinen, T., Alfaro-Cervello, C., Bonnaud, F., Miguez, A., Benhaim, L., and Xu, Y. (2011). Vascular endothelial growth factor receptor 3 directly regulates murine neurogenesis. Genes Dev. *25*, 831–844.

Cho, G., Lim, Y., Cho, I.T., Simonet, J.C., and Golden, J.A. (2014). Arx together with FoxA2, regulates Shh floor plate expression. Dev. Biol. *393*, 137–148.

Codega, P., Silva-Vargas, V., Paul, A., Maldonado-Soto, A.R., DeLeo, A.M., Pastrana, E., and Doetsch, F. (2014). Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche. Neuron *82*, 545–559.

Dromard, C., Guillon, H., Rigau, V., Ripoll, C., Sabourin, J.-C., Perrin, F., Scamps, F., Bozza, S., Sabatier, P., and Lonjon, N. (2008). Adult human spinal cord harbors neural precursor cells that generate neurons and glial cells in vitro. J. Neurosci. Res. *86*, 1916–1926.

Fiorelli, R., Cebrian-Silla, A., Garcia-Verdugo, J.M., and Raineteau, O. (2013). The adult spinal cord harbors a population of GFAP-positive progenitors with limited self-renewal potential. Glia *61*, 2100–2113.

Fu, H., Qi, Y., Tan, M., Cai, J., Hu, X., Liu, Z., Jensen, J., and Qiu, M. (2003). Molecular mapping of the origin of postnatal spinal cord ependymal cells: evidence that adult ependymal cells are derived from Nkx6. 1+ ventral neural progenitor cells. J. Comp. Neurol. *456*, 237–244.

Gage, F.H., and Temple, S. (2013). Neural stem cells: generating and regenerating the brain. Neuron *80*, 588–601.

Garcia-Ovejero, D., Arevalo-Martin, A., Paniagua-Torija, B., Florensa-Vila, J., Ferrer, I., Grassner, L., and Molina-Holgado, E. (2015). The ependymal region of the adult human spinal cord differs from other species and shows ependymoma-like features. Brain *138*, 1583–1597.

Grebbin, B.M., Hau, A.-C., Groß, A., Anders-Maurer, M., Schramm, J., Koss, M., Wille, C., Mittelbronn, M., Selleri, L., and Schulte, D. (2016). Pbx1 is required for adult SVZ neurogenesis. Development *143*, 2281–2291.

Habib, N., Li, Y., Heidenreich, M., Swiech, L., Avraham-Davidi, I., Trombetta, J.J., Hession, C., Zhang, F., and Regev, A. (2016). Div-Seq: single-nucleus RNA-Seq reveals dynamics of rare adult newborn neurons. Science *353*, 925–928.

Hamilton, L., Truong, M., Bednarczyk, M., Aumont, A., and Fernandes, K. (2009). Cellular organization of the central canal ependymal zone, a niche of latent neural stem cells in the adult mammalian spinal cord. Neuroscience *164*, 1044–1056.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2008). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57.

Ingram, W., McCue, K., Tran, T., Hallahan, A., and Wainwright, B. (2008). Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling. Oncogene *27*, 1489–1500.

Jarvelainen, H., Sainio, A., and Wight, T.N. (2015). Pivotal role for decorin in angiogenesis. Matrix Biol. *43*, 15–26.

Kang, P., Lee, H.K., Glasgow, S.M., Finley, M., Donti, T., Gaber, Z.B., Graham, B.H., Foster, A.E., Novitch, B.G., and Gronostajski, R.M. (2012). Sox9 and NFIA coordinate a transcriptional regulatory cascade during the initiation of gliogenesis. Neuron *74*, 79–94.

Khazanov, S., Paz, Y., Hefetz, A., Gonzales, B.J., Netser, Y., Mansour, A.A., and Ben-Arie, N. (2017). Floor plate descendants in the ependyma of the adult mouse central nervous system. Int. J. Dev. Biol. *61*, 257–265.

Kubota, H., Avarbock, M.R., and Brinster, R.L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. U S A *101*, 16489–16494.

Lallemand, Y., Moreau, J., Saint Cloment, C., Vives, F.L., and Robert, B. (2013). Generation and characterization of a tamoxifen inducible Msx1CreERT2 knock-in allele. Genesis *51*, 110–119.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J., et al. (2007). Genome-wide atlas of gene expression in the adult mouse brain. Nature *445*, 168–176.

Llorens-Bobadilla, E., and Martin-Villalba, A. (2017). Adult NSC diversity and plasticity: the role of the niche. Curr. Opin. Neurobiol. *42*, 68–74.

Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwadlo, K., and Martin-Villalba, A. (2015). Single-cell transcriptomics reveals a population of dormant neural stem cells that become activated upon brain injury. Cell Stem Cell *17*, 329–340.

Marichal, N., Reali, C., Trujillo-Cenóz, O., and Russo, R.E. (2017). Spinal cord stem cells in their microenvironment: the ependyma as a stem cell niche. Adv. Med. Exp. Biol. *1041*, 55–79.

Mo, J.S., Park, H.W., and Guan, K.L. (2014). The Hippo signaling pathway in stem cell biology and cancer. EMBO Rep. *15*, 642–656.

Mothe, A.J., Zahir, T., Santaguida, C., Cook, D., and Tator, C.H. (2011). Neural stem/progenitor cells from the adult human spinal cord are multipotent and self-renewing and differentiate after transplantation. PLoS One *6*, e27079.

Nolte, C., Matyash, M., Pivneva, T., Schipke, C.G., Ohlemeyer, C., Hanisch, U.K., Kirchhoff, F., and Kettenmann, H. (2001). GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue. Glia *33*, 72–86.

Petracca, Y.L., Sartoretti, M.M., Di Bella, D.J., Marin-Burgin, A., Carcagno, A.L., Schinder, A.F., and Lanuza, G.M. (2016). The late and dual origin of cerebrospinal fluid-contacting neurons in the mouse spinal cord. Development *143*, 880–891.

Pfenninger, C.V., Steinhoff, C., Hertwig, F., and Nuber, U.A. (2011). Prospectively isolated CD133/CD24-positive ependymal cells from the adult spinal cord and lateral ventricle wall differ in their long-term in vitro self-renewal and in vivo gene expression. Glia *59*, 68–81.

Prosser, H.M., Bradley, A., and Caldwell, M.A. (2007). Olfactory bulb hypoplasia in Prokr2 null mice stems from defective neuronal progenitor migration and differentiation. Eur. J. Neurosci. *26*, 3339–3344.

Ramos, C., and Robert, B. (2005). msh/Msx gene family in neural development. Trends Genet. *21*, 624–632.

Ren, Y., Ao, Y., O'Shea, T.M., Burda, J.E., Bernstein, A.M., Brumm, A.J., Muthusamy, N., Ghashghaei, H.T., Carmichael, S.T., and Cheng, L. (2017). Ependymal cell contribution to scar formation after spinal cord injury is minimal, local and dependent on direct ependymal injury. Sci. Rep. *7*, 41122.

Rosenberg, A.B., Roco, C.M., Muscat, R.A., Kuchina, A., Sample, P., Yao, Z., Graybuck, L.T., Peeler, D.J., Mukherjee, S., and Chen, W. (2018). Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. Science *360*, 176–182.

Ruzinova, M.B., and Benezra, R. (2003). Id proteins in development, cell cycle and cancer. Trends Cell Biol. *13*, 410–418.

Sabourin, J.C., Ackema, K.B., Ohayon, D., Guichet, P.O., Perrin, F.E., Garces, A., Ripoll, C., Charité, J., Simonneau, L., and Kettenmann, H. (2009). A mesenchymal-like ZEB1+ niche harbors dorsal radial glial fibrillary acidic protein-positive stem cells in the spinal cord. Stem Cells *27*, 2722–2733.

Smith, E., Hargrave, M., Yamada, T., Begley, C.G., and Little, M.H. (2002). Coexpression of SCL and GATA3 in the V2 interneurons of the developing mouse spinal cord. Dev. Dyn. *224*, 231–237.

Stenudd, M., Sabelström, H., and Frisén, J. (2015). Role of endogenous neural stem cells in spinal cord injury and repair. JAMA Neurol. *72*, 235–237.

Tomer, R., Ye, L., Hsueh, B., and Deisseroth, K. (2014). Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat. Protoc. *9*, 1682.

Ward, M., and Cunningham, A. (2015). Developmental expression of vascular endothelial growth factor receptor 3 and vascular endothelial growth factor C in forebrain. Neuroscience *303*, 544–557.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., and Reynolds, B.A. (1996). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J. Neurosci. *16*, 7599–7609.

Wilson, L., and Maden, M. (2005). The mechanisms of dorsoventral patterning in the vertebrate neural tube. Dev. Biol. *282*, 1–13.

Xing, L., Anbarchian, T., Tsai, J.M., Plant, G.W., and Nusse, R. (2018). Wnt/β-catenin signaling regulates ependymal cell development and adult homeostasis. Proc. Natl. Acad. Sci. U S A *115*, E5954–E5962.

Xu, W., Sachewsky, N., Azimi, A., Hung, M., Gappasov, A., and Morshead, C.M. (2017). Myelin basic protein regulates primitive and definitive neural stem cell proliferation from the adult spinal cord. Stem Cells *35*, 485–496.

Zeisel, A., Hochgerner, H., Lonnerberg, P., Johnsson, A., Memic, F., van der Zwan, J., Haring, M., Braun, E., Borm, L.E., La Manno, G., et al. (2018). Molecular architecture of the mouse nervous system. Cell *174*, 999–1014.e22.

Stem Cell Reports, Volume *12*

Supplemental Information

RNA Profiling of the Human and Mouse Spinal Cord Stem Cell Niches Reveals an Embryonic-like Regionalization with MSX1^{+} Roof-Plate-Derived Cells

Hussein Ghazale, Chantal Ripoll, Nicolas Leventoux, Laurent Jacob, Safa Azar, Daria Mamaeva, Yael Glasson, Charles-Felix Calvo, Jean-Leon Thomas, Sarah Meneceur, Yvan Lallemand, Valérie Rigau, Florence E. Perrin, Harun N. Noristani, Brenda Rocamonde, Emmanuelle Huillard, Luc Bauchet, and Jean-Philippe Hugnot

Supplemental Information

Fig S1: Allen brain atlas validation. **Related to Figure 3 and table S1. A:** Examples of genes found enriched in the spinal cord EZ in the present study which are also validated in the Allen brain atlas based on in situ hybridization. **B:** Examples of transcription factors found enriched in the spinal cord EZ which are also validated in the Allen brain atlas. For *Pax6*, the Gensat Atlas image (based on transgenic mice) is presented. The black arrow indicates ventral *Pax6* negative cells.

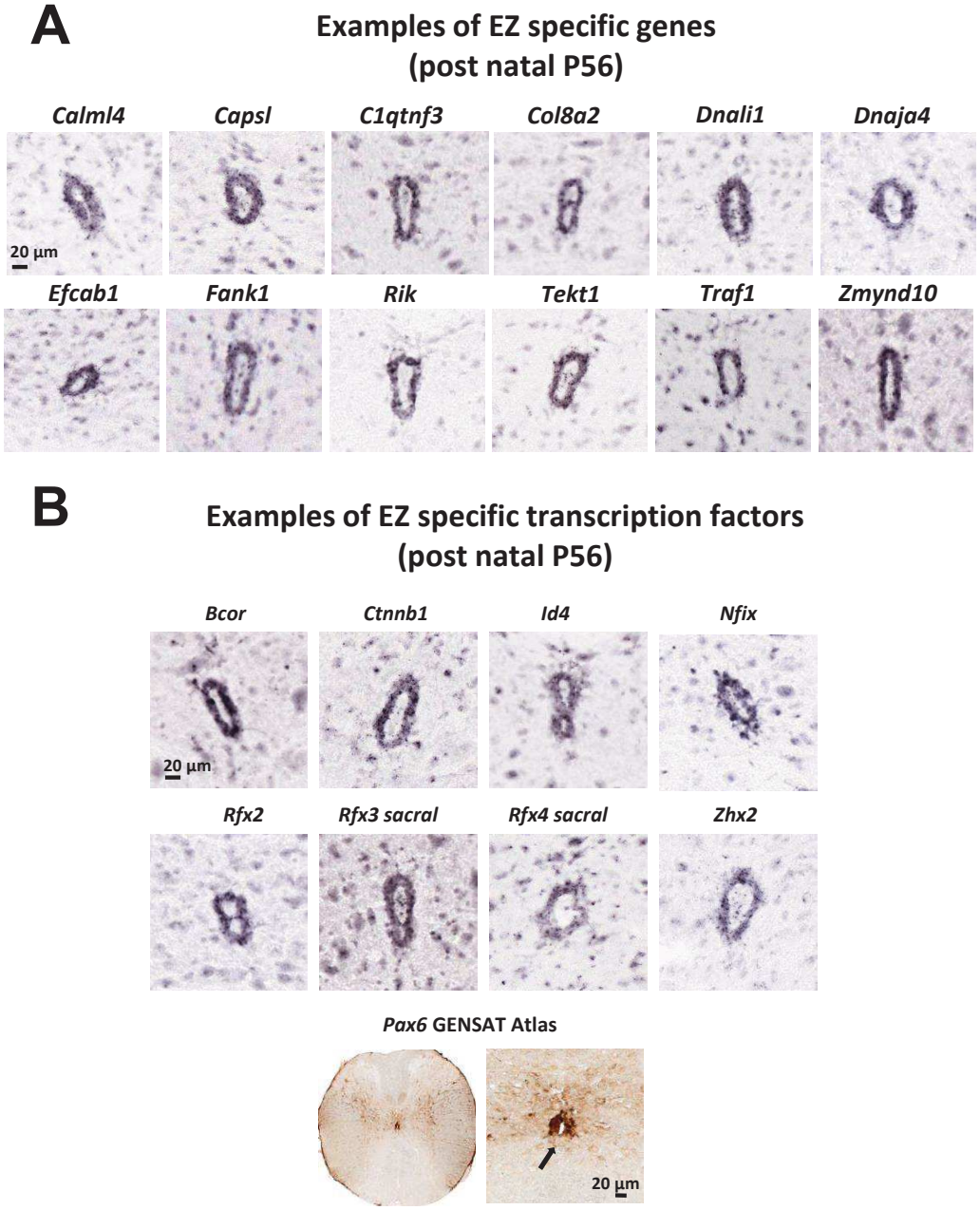


Fig S2: Characterization of ARX$^+$, DCN$^+$, FOXA2$^+$ and TAL1$^+$ cells in the mouse EZ. Related to Figure 3. Co-IF show that DCN, TAL1 and FOXA2 are expressed by CSF-N detected by PKD2L1 staining. ARX/PKD2L1 antibody incompatibility precluded double labeling, however ventral ARX$^+$ cells are distinct from FOXA2$^+$ and DCN$^+$ cells and are thus unlikely to be CSF-N. Presented images are representative of at least 10 sections per animal (3 mice analyzed). Nuclei (blue) are stained with Hoechst. Scale bar, shown on first image, is the same for all images. Insets at the bottom are high magnification of boxed areas.

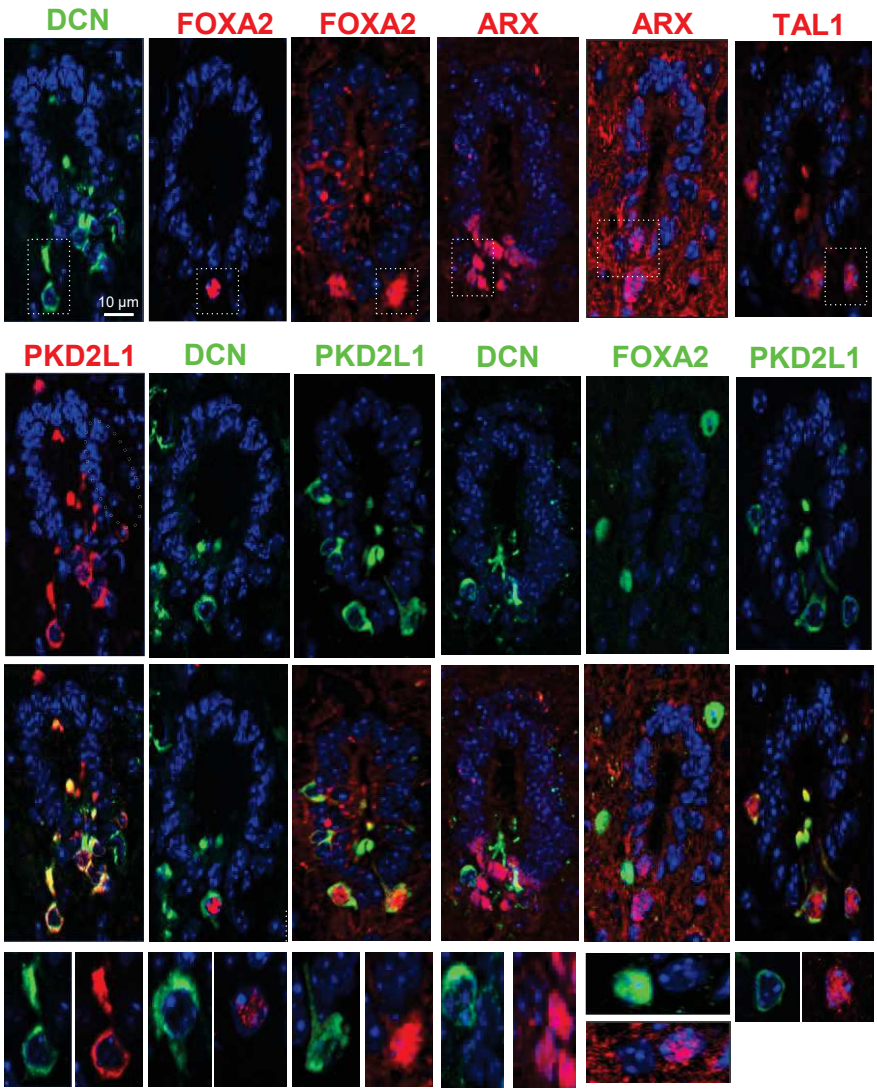


Fig S3: Atlas validation of regionalized gene expression. Related to Figure 3 and Table S5. A: Allen brain atlas examples of dorsally and ventrally expressed genes identified in table S5**. B:** Validation of expression of *Msx1* in the dorsal EZ in Allen brain (post-natal P4) and Gensat atlases. EZ region is shown by dotted line oval shape.

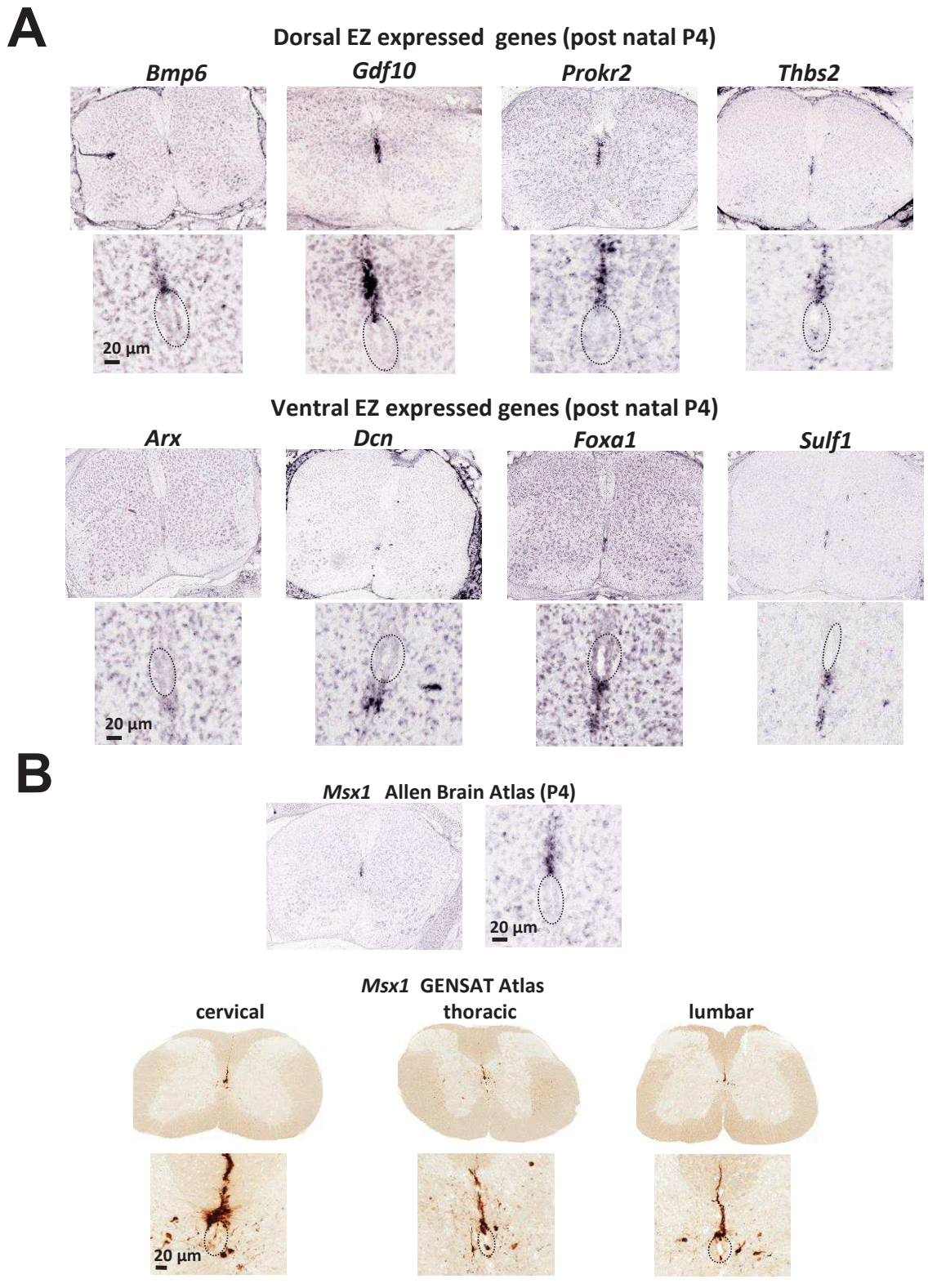


Fig S4: human EZ characterization. Related to Figure 1 and 2. A: IF for the indicated proteins in the human EZ (second patient aged 46, lumbar level). As often observed in aged human tissues, this second sample presented non-specific red autofluorescence aggregates (most likely lipofuscin) which are observed with the control antibody. White arrowheads show negative cells indicative of the staining specificity. Images are oriented with ventral part at bottom. These images are representative of 4 sections. **B:** Co-localization of FOXA2 and ARX in the ventral part of the human EZ (patient aged 17, n= 4 sections).

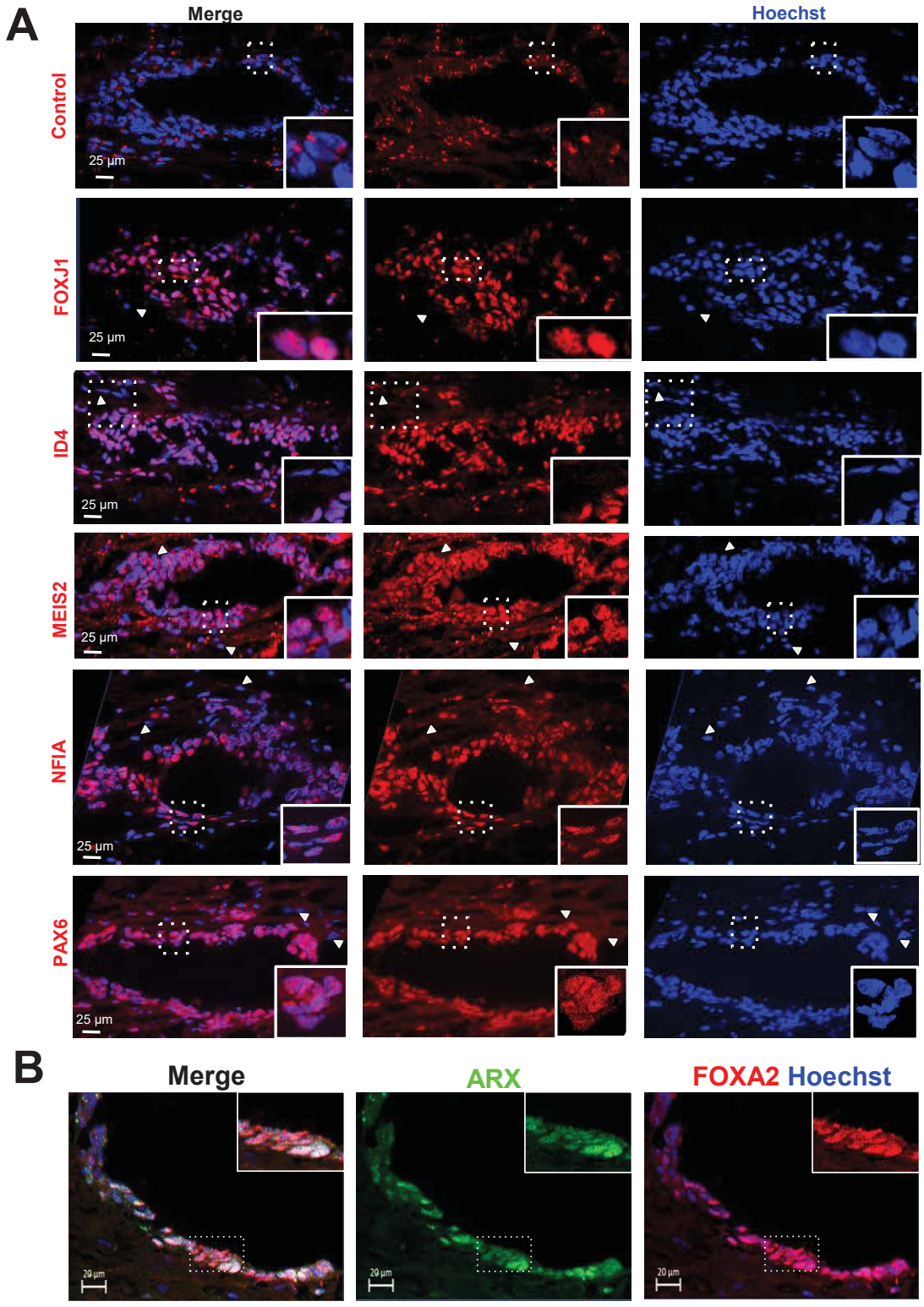
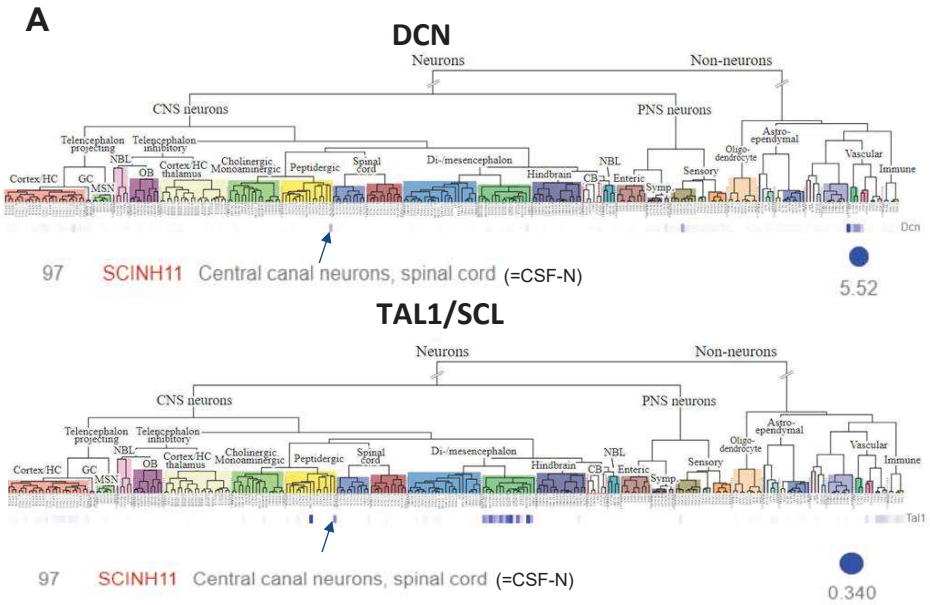
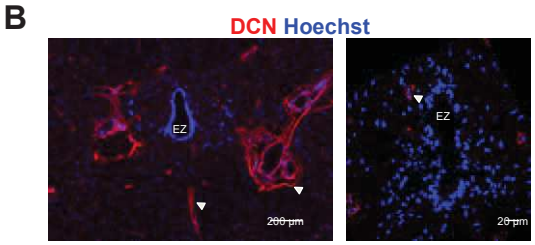


Fig S5: Comparison with single cell databases. **Related to Figure 2 and 3. A:** Single cell RNA expression confirms that *Tal1/Scl* and *Dcn* are expressed by CSF-N in the spinal cord. These data were obtained from the website (http://mousebrain.org/) associated to this publication (Zeisel et al, Cell. 2018 Aug 9;174(4):999-1014). Adult cell subtypes showing *Tal1* and *Dcn* expression are indicated by a blue rectangle and arrows point to the spinal cord CSF-N population (referred to as SCINH11 in Zeisel's article). Quantitative expression for these cells is presented as a blue circle and underneath value. **B:** Immunofluorescence for DECORIN (DCN) in the human spinal cord EZ (17-year-old patient) showing highly stained vessels (arrowheads) but no labeling in the EZ. **C:** Fold change expression (EZ/peri EZ), retrieved from our study, of 6 mouse CSF-N markers in mouse and human samples (17 and 46-year-old; (y.o.)). Compared to the mouse EZ, no enrichment is observed for these markers in the human EZ. **D:** Single cell RNA expression shows that *Msx1, Id4, BMP6 and GDF10/BMP3b* are mainly expressed in quiescent neural stem cells in the brain SVZ. These data were retrieved from the website (https://martin-villalba-lab.shinyapps.io/scRNAseq_CSC2015/) associated to this publication (Llorens-Bobadilla E et al Cell Stem Cell. 2015 Sep 3;17(3):329-40).





C

CSF-N markers	Fold change (EZ/peri EZ)		
	Mouse	Human (17 y.o.)	Human (46 y.o.)
Pkd2l1	63,3	0,9	0,9
Dcn	7,1	1,1	1,1
Pdzk1ip1	6,0	1,1	1,1
Crct1	3,2	1,0	1,0
Espn	2,3	1,0	1,0
Pkd1l2	1,5	0,8	0,8

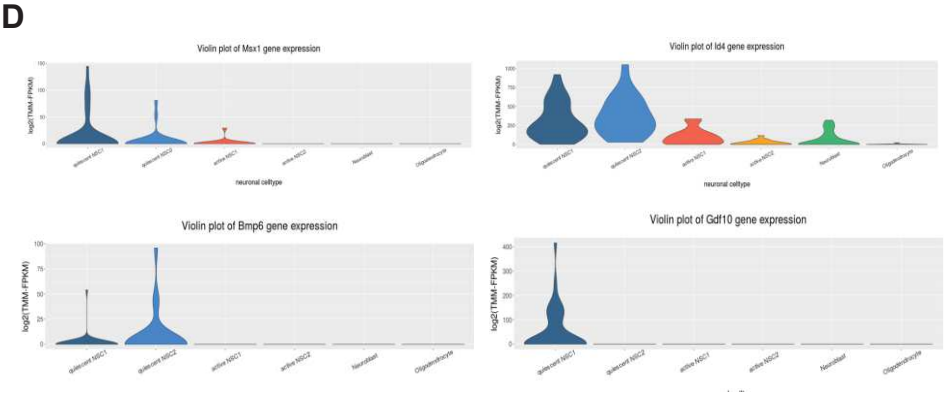
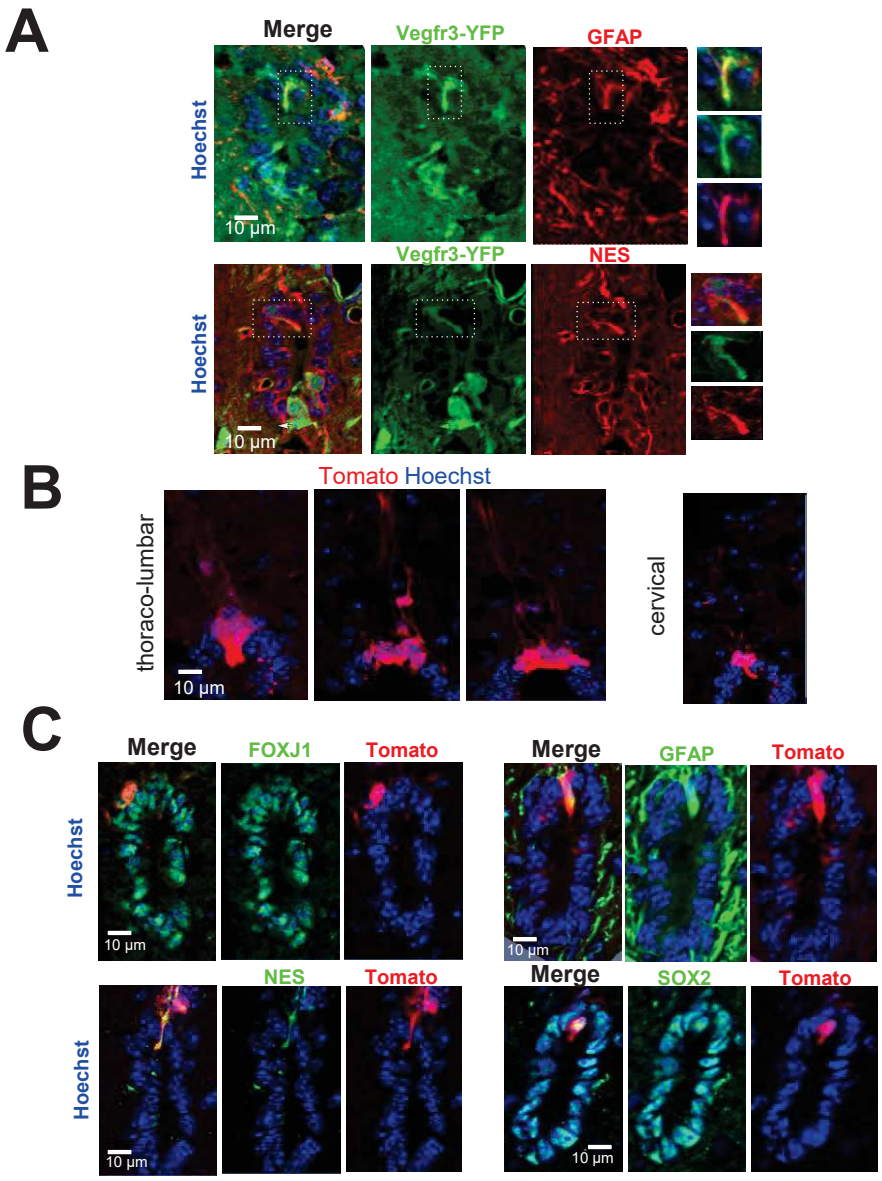


Fig S6: Mouse EZ characterization. Related to Figure 4. A: Characterization of EZ YFP$^+$ cells in the Vegfr3-YFP mice. IF for GFAP and NES (nestin), show that dorsal YFP$^+$ express these two intermediate filaments. Co-localization of GFAP and NES in the ventral YFP$^+$ cells were ambiguous and inconclusive. These images are representative of 10 sections per animal (3 mice analyzed). **B and C: Characterization of dorsal Tomato$^+$ cells in Msx1-CreERT2/Rosa-Tomato mice**. **B:** examples of clusters of Tomato$^+$ cells found in the EZ roof at the cervical and thoracic-lumbar spinal cord levels. **C:** IF for indicated proteins showing co-expression in Tomato$^+$ cells. Presented images are representative of 10 sections per animal (3 mice analyzed).



Supplemental Tables

Table S1: mouse EZ genes (Excel file). This file includes 3 lists of genes whose expression is enriched in the mouse adult EZ (fold change ≥2.0, n=4 mice) (full list + transport-related genes + transcription-related genes) + analyses (3 GO terms, KEGG pathway, upkeywords) + bar plots of GO analyses. Genes belonging to the solute carrier family (*Scl*) are in red in the transport-related spreadsheet.

Table S2: human EZ genes (Excel file). This file includes 3 lists of genes whose expression is enriched in the human EZ (fold change ≥2.0, n=2 samples,17- and 46-year-old) (full list + transport-related genes + transcription-related genes) + analyses (3 GO terms, KEGG pathway, upkeywords) + bar plots of GO analyses. Genes belonging to the solute carrier family (*Scl*) are in red in the transport-related spreadsheet.

Table S3: Comparison of mouse and human EZ-enriched genes (Excel file). This file includes 3 lists of genes whose expression is enriched in the human and mouse EZ (fold change ≥2.0) (full list + transport-related genes + transcription-related genes) + analyses (3 GO terms, KEGG pathway, upkeywords) + bar plots of GO analyses. The gene lists also include columns showing genes which are more specifically enriched in one specie.

Table S4: Comparison with spinal cord ependymal and CSF-N specific genes identified in two other studies (Excel file). This table shows genes which are commonly identified in our study and in these two other studies: **1-**Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding" *Science*. 2018 Apr 13;360(6385):176-182 and **2-**Molecular architecture of the mouse nervous system. *Cell*. 2018 Aug 9;174(4):999-1014). Genes which are identified in the three studies are highlighted in green, those identified in two studies including ours are in red.

Table S5: Dorsal, lateral and ventral EZ-enriched genes (Excel file). This file shows 3 lists of genes which are specifically enriched in the different parts of the mouse adult EZ (n=4 mice).

Table S6: Antibody list

Name	Species	Supplier	Reference	Dilution for IF
ARX (human sections)	sheep	R&D	AF7068	1/2000
ARX (mouse sections)	Rabbit	Gift from Pr Morohashi, (Kyushu University, Japan)		1/500
b-CATENIN	Mouse	BD Bioscience	610153	1/500
CD24	Mouse	eBioscience	12-0241	1/500
C-RET	Goat	R&D	AF482	1/500
DCN	Goat	R&D	AF1060	1/100
FOXJ1	Mouse	eBioscience	14-9965-82	1/100
FOXA2	Goat	R&D	AF2400	1/100
FOXA2	Rabbit	Abcam	ab108422	1/300
GFAP	Rabbit	Dako	Z0334	1/1000
GFP	Rabbit	Abcam	ab183734	1/500
GFP	Chicken	Abcam	ab13970	1/2000
ID4	Rabbit	Biocheck	BCH-9/82-12	1/500
MEIS2	Rabbit	Abcam	ab73164	1/200
MSX1	Goat	R&D	AF5045	1/400
NESTIN	Chicken	Abcam	ab81755	1/1000
NFIA	Rabbit	Atlas Ant.	HPA006111	1/200
PAX6	Rabbit	Covance	PRB-278P	1/500
PBX1	Rabbit	Cell signaling technology	4342	1/400
PKD2L1	Rabbit	Millipore	AB9084	1/1000
SOX2	Rabbit	Cell signaling technology	23064S	1/400
SOX4	Rabbit	Abcam	ab86809	1/400
SOX9	Rabbit	Cell signaling technology	82630S	1/400
SOX11	Rabbit	Atlas Ant.	HPA000536	1/500
TAL1	Goat	Santa-cruz	sc-12984	1/250

Supplementary Data Related to the paper

Materials and Methods

Spinal cord neural stem cell cultures

Neurosphere cultures were derived from adult mouse spinal cords using the protocol and medium detailed in (Hugnot, 2013). Cell growth (fig 6I and 7I) was measured by seeding dissociated cells (1000 or 5000 cells per well) in 1 ml of media in 24-well plates coated with poly-HEMA (Sigma P3932) to inhibit cell adherence. After 5 or 7 days, the NS were directly dissociated by addition of trypsin in the wells (0.5% final) and the cell number was measured with an automated cell counter (Z2, Beckman Coulter). To determine the percentage of NS formation at clonal density, Tomato+ and Tomato- cells were seeded at 1 cell/well in 96-well plates using an automatic cell seeding device (Aria cytometer BD). After two weeks, the number of spheres and their size was visually determined. To assess the differentiation of adult spinal cord stem cells, NS were enzymatically dissociated and seeded on poly-D-lysine/laminin-coated coverslips with medium containing 2% serum without growth factor. After 4 days, the coverslips were fixed for 20 min with 4% formaldehyde PBS-solution and processed for IF. For EdU incorporation (fig 6I, 7I), cells were incubated with EdU 10 µM for 3 hours and processed for staining following manufacturer's recommendation (Baseclick kit).

Virus

To access the effect of MSX1 overexpression on neural stem cells, inducible lentiviruses combining an EGFP-T2A-Puromycine resistance gene and the coding sequence for mouse *Msx1* or *luciferase*, controlled by a Tre3G promoter, were built (Vectorbuilder). A third lentivirus was built to express a blasticidin resistance gene and the doxycycline-regulated Tet3G activator. Growing spinal cord NS were co-infected with luciferase or Msx1 lentiviruses and Tet3G virus and selected for 2 weeks with blasticidin (0,5 µg/ml) and puromycine (0,2 µg/ml). The expression of MSX1 or luciferase was induced by adding doxycycline 1 µg/ml in the medium. To access the effect of ID4 on neural stem cells, cells were infected with a retrovirus combining expression of GFP with and without ID4 and a blasticidin resistance gene (gift from Dr E Huillard's lab, ICM, Paris). Cells were selected for 2 weeks with blasticidin 0.5 µg/ml before accessing their growth (fig 7I). For activated RAS overexpression (fig 7), we used a lentivirus containing a K-RAS (G412 mutation) cDNA under the control of the PGK promoter (Addgene #35633).

QPCR

RNA was extracted from growing or differentiated spinal cord NS using RNeasy kit (Qiagen). cDNA was obtained by reverse transcription with random hexamers and reverse transcriptase (Superscript II, Promega). Quantitative PCR were performed using a Sybr PCR kit (Kapa) and a LightCycler 480 apparatus (Roche). Primers sequences: Id4 (GTTCACGAGCATTCACCGTA& AAGGTTGGATTCACGATTGC); GAPDH (TGTCCGTCGTGGATCTGAC & CCTGCTTCACCACCTTCTTG).

Western blot (WB)

Protein extraction and western blot were performed according to classical procedures. Cells were collected and lysed in RIPA buffer (Sigma) containing proteases- and phosphatase inhibitors (Roche). Protein concentration was determined using a protein assay kit (Biorad). Proteins (20 µg) were separated by SDS-PAGE, and transferred onto PVDF membranes (Biorad). Peroxidase secondary antibodies (Jackson Immunoresearch Laboratory), ECL kit (Biorad) and ChemiDoc apparatus were used for revelation.

Results

Limited NS formation of MSX1+ roof cells and negative influence of MSX1 on proliferation of spinal cord neural stem cells

Multipotent NS can be derived from the adult EZ (Barnabé-Heider et al., 2010; Becker et al., 2018). Reminiscent of the situation in the SVZ stem cell niche, we investigated whether the Tomato+ roof cells were able togenerate NS as several types of cells in the spinal cord niche do. Attempts to generate NS directly from a limited number of Tomato+ cells obtained using fluorescence-activated cell sorting (FACS) of dissociated spinal cords were negative (n=4, 50-100 cells each experiment). We then performed NS cultures from unsorted cells, directly dissociated from Msx1-Tomato mouse spinal cord. After one week, observation of NS grown at low density and derived from a single Msx1-tomato mouse showed that the vast majority of NS (diameter > 200 µm) were Tomato negative thus indicating that Tomato+ cells are not the main NS-forming cells in the EZ. Careful examination of the culture over days indicates that most Tomato+ cells remained assingle cells or generated small NS (<50 µm). However, we constantly observed that a small number of NS with a size > 200 µm (3.4% of total NS, n=3 independent experiments) were completely red (fig 1'A) and grew larger over days. After 10 days of culture, we used FACS to purify the Tomato+ cells and reseeded them. These cells were able to form new NS which could be propagated for 7 passages without any sign of reduced proliferation (fig 1'B). When seeded as single cells in a 96 well plate, these $Tomato^+$ cells formed new NS (diameter > 300 µm) with a frequency of 7.6% which is similar to the frequency observed with $Tomato^-$ cells (11%). Characterization of $Tomato^+$ NS by WB and IF indicated that they express proteins typically found in neural stem cells such as SOX2 (fig 1'C) and VCAM1 (Kokovay et al., 2012) (fig 1'G). We next tested the multipotentiality of $Tomato^+$ NS cells by placing them in differentiation condition. In this situation, we observed the formation of cells with different morphologies (fig 1'D) and expressing markers typical for astrocytes (GFAP), neurons (MAP2, DCX, βIII TUB) and oligodendrocytes (CNPase, OLIG2) (fig 1'E, F) which demonstrates the ability of the $Tomato^+$ cells to generate several cell types.

Msx1 expression is positively-regulated by BMP morphogens during development (Ramos and Robert, 2005). The strong expression of *Bmp6 and Gdf10/Bmp3b* transcripts in the dorsal EZ (fig S3A) led us to explore whether the roof MSX1+ cells co-expressed these morphogens in vivo. However we were unable to obtain convincing staining by IF, possibly due to the limited abundance of these morphogens. In contrast, *in vitro*, BMP6 and GDF10 proteins were readily

detected by WB in NS derived from Tomato+ cells (fig 1'G). These Tomato+ cells also expressed a low level of MSX1 protein (fig 1'H) and this can be further increased by adding BMP6 in the media (10 ng/ml), as evidenced by WB and IF. As previously reported for BMPs (Martynoga et al., 2013; Sabourin et al., 2009), BMP6 addition also led to a drastic reduction of proliferation (fig 2'H).

Next, the absence or low proliferation of roof MSX1+ cells in vivo (fig 5G) and the increase of MSX1 (fig 1'H) concomitantly with the reduction of proliferation induced by BMP6 (fig 2'H) in vitro prompted us to question the influence of MSX1 on spinal cord neural stem cells. We designed two inducible-lentiviruses in which MSX1 or a control gene (luciferase) can be tightly regulated by doxycycline (fig S1'A). Compared to the luciferase virus, induction of MSX1 in growing NS derived from adult spinal cord led to a sharp reduction of cell number after 5 days of growth, which was associated with a reduced proliferation rate as evidenced by EdU incorporation (fig 1'I). The influence of MSX1 in NS placed in differentiation condition was also explored. We observed that induction of MSX1 led to a reduction of OLIG2+, DCX+ and MAP2+ cells while the fraction of GFAP+ astrocytic cells was increased (fig S1'B).

Taken together, these data indicate that MSX1+ cells have a low potential to form NS and that MSX1 overexpression reduces neural stem cell proliferation and promote astrocytic differentiation.

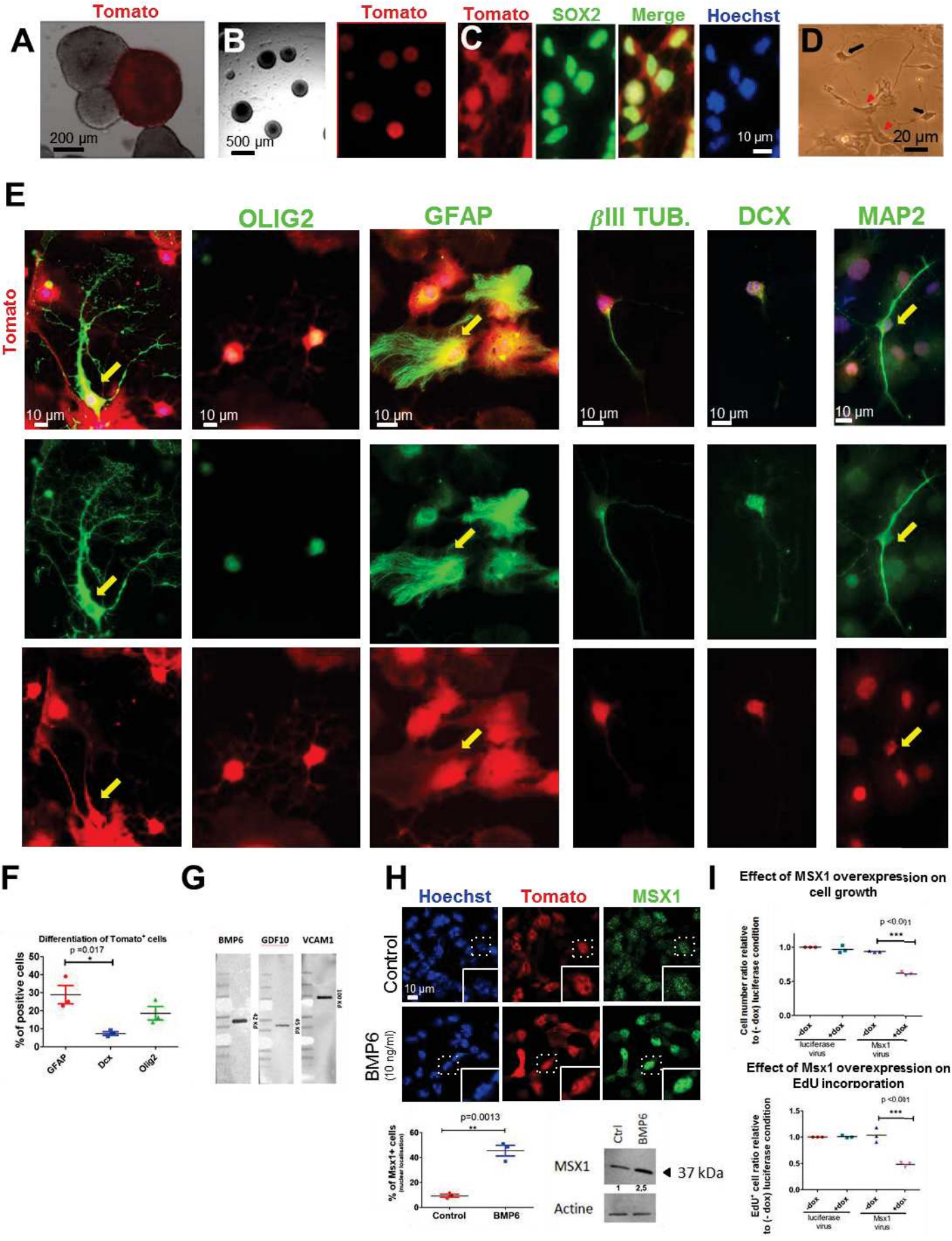

A
Tomato
200 µm
B
500 µm
Tomato
C
Tomato
SOX2
Merge
Hoechst
10 µm
D
20 µm
E
OLIG2
GFAP
βIII TUB.
DCX
MAP2
Tomato
10 µm
F
Differentiation of Tomato+ cells
p =0.017
% of positive cells
GFAP
Dcx
Olig2
G
BMP6
GDF10
VCAM1
H
Hoechst
Tomato
MSX1
Control
BMP6
(10 ng/ml)
10 µm
p=0.0013
% of Msx1+ cells
Control
BMP6
Ctrl
BMP6
MSX1
37 kDa
Actine
I
Effect of MSX1 overexpression on cell growth
p <0.001
Cell number ratio relative to (- dox) luciferase condition
-dox
+dox
luciferase virus
Msx1 virus
Effect of Msx1 overexpression on EdU incorporation
p <0.001
EdU+ cell ratio relative to (- dox) luciferase condition

Figure 1': In vitro properties of Tomato+ cells. A: Tomato+ neurosphere (size > 200 μm) observed in a spinal cord culture (P0) derived from Msx1-CreERT2/Rosa-Tomato mouse. **B:** FACS sorted Tomato+ cells formed new NS which can be propagated for at least 7 passages. **C:** IF for SOX2 on Tomato+ cells. **D:** After differentiation**,** Tomato+ NS generate cells with different morphologies (black and red arrows). **E:** IF analysis with indicated markers of cells obtained after differentiation of Tomato+ neurospheres. Yellow arrows indicate double positive cells. **F:** Quantification of differentiation of Tomato+ cells (average of % of positive cells +/- S.E.M, n=3 independent experiments, test=one-way ANOVA + Tukey posttest). **G:** Detection of BMP6, GDF10 and VCAM1 by WB in proteins extracted from Tomato+ neurospheres. **H:** *Upper panels*: Detection of MSX1 by IF in growing Tomato+ cells without and with BMP6 10ng/ml. *Lower left panel:* Average % +/- S.E.M of cells showing a nuclear strong expression of MSX1 (n=3 independent experiments, unpaired t-test). Weak cytoplasmic detection of MSX1 could be observed in untreated cells but was not quantified. *Lower right panel:* Detection of MSX1 by WB in cells with and without BMP6 10 ng/ml. Numbers show relative quantification of Msx1 signal normalized by actin. **I:** Effect of MSX1 overexpression on spinal cord stem cell growth (n=3 independent experiments, test=one-way ANOVA + Tukey posttest). Upper diagram shows the cell number ratio +/- S.E.M obtained after 5 days with and without doxycyclin in cells infected with Msx1 or control (luciferase) lentivirus. Lower panel shows the ratio of EdU+ cells +/- S.E.M (3 hour EdU incorporation) obtained in the different conditions.

ID4 expression is negatively regulated by RAS pathways and positively by BMP6 in spinal cord stem cells

ID proteins are important proteins controlling stem cell fate (Ruzinova and Benezra, 2003). The identification of a conserved and specific expression of ID4 in EZ cells and the even higher expression observed in dorsal MSX1+ quiescent cells (fig 3) led us to explore how ID4 was expressed in adult spinal cord NS. Unexpectedly, ID4 protein was barely expressed in growing NS but sharply increased after differentiation when co-localized with GFAP+ astrocytic cell but not cells (fig 2'A, B, C). This increase in ID4 was reversible as reintroduction of the EGF/FGF2 growth factors in the culture reduced ID4 expression at the protein and RNA levels (fig 2'A, B). This suggested that ID4 expression was negatively regulated by pathways downstream of EGF/FGF2 most likely by the MAPK/ERK signaling. We tested this hypothesis by infecting differentiated cells with lentiviruses expressing activated-RAS or luciferase as a control. As expected RAS virus led to strong phosphorylation of ERK (fig 2'D) and cell cycle re-entry, as evidenced by the presence of proliferating cells and the sharp increase of KI67+ cells (fig 2'E). ID4 was strongly reduced in these cultures compared to control virus infected cells (fig 2'F). After one week, the RAS-infected cultures spontaneously generated NS which can be grown and passaged in the absence of growth factors (fig 2'G). When placed in differentiating conditions in the presence of serum, these cells have a reduced ID4 level compared to control NS thus indicating that activated RAS inhibits ID4 expression (fig 2'G).

Next, as ID proteins are positively regulated by BMP proteins (Ruzinova and Benezra, 2003), we tested the influence of BMP6 on ID4 in growing NS. This resulted in ID4 induction and as mentioned previously, in reduction of KI67+ cells (fig 2'H). Finally, we explored the influence of ID4 on spinal cord stem cell growth by overexpressing it in growing NS using a retrovirus approach (fig S1'C). As shown on (figure 2'I), a reduction in cell number was observed which was concomitant with a decrease in EdU incorporation. It could be hypothesized that this could be accompanied by an astrocytic differentiation of the cells, however, conversely, WB analysis in growing spinal cord NS infected with ID4 virus showed a reduction of GFAP compared to control virus (fig S1'D). Collectively, these results identified ID4 as a negative regulator of spinal cord stem cell proliferation which is tightly regulated by MAPK/ERK and BMP signaling.

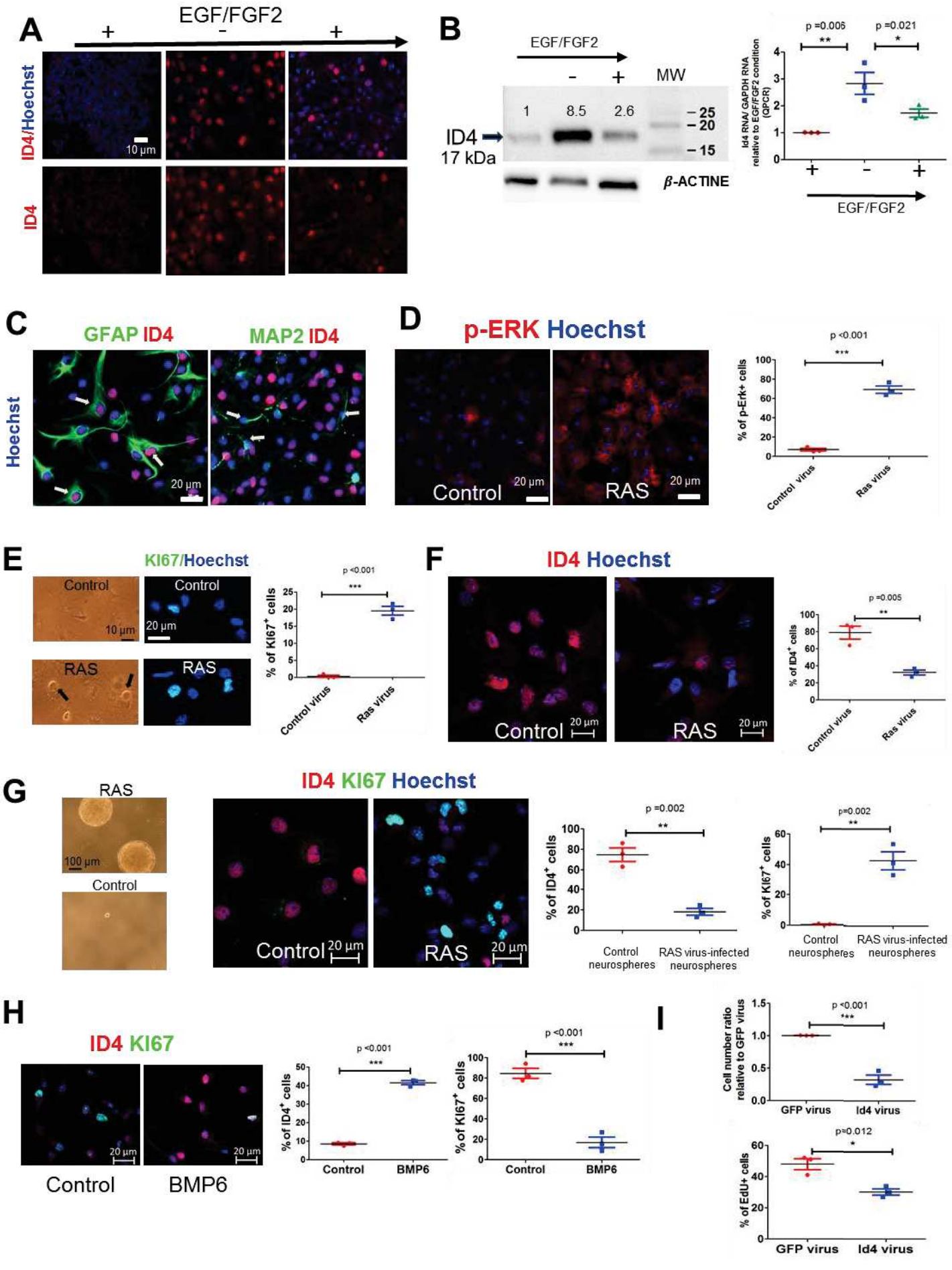

A
EGF/FGF2
+
-
+
ID4/Hoechst
ID4
10 µm
B
EGF/FGF2
- + MW
1 8.5 2.6
ID4
17 kDa
25
20
15
β-ACTINE
p =0.006
p =0.021
Id4 RNA/ GAPDH RNA relative to EGF/FGF2 condition (QPCR)
EGF/FGF2
C
GFAP ID4
MAP2 ID4
Hoechst
20 µm
D
p-ERK Hoechst
Control
RAS
20 µm
p <0.001
% of p-Erk+ cells
Control virus
Ras virus
E
KI67/Hoechst
Control
RAS
10 µm
20 µm
p <0.001
% of KI67+ cells
Control virus
Ras virus
F
ID4 Hoechst
Control
RAS
20 µm
p =0.005
% of ID4+ cells
Control virus
Ras virus
G
RAS
Control
100 µm
ID4 KI67 Hoechst
Control
RAS
20 µm
p =0.002
% of ID4+ cells
Control neurospheres
RAS virus-infected neurospheres
p=0.002
% of KI67+ cells
Control neurospheres
RAS virus-infected neurospheres
H
ID4 KI67
20 µm
Control
BMP6
p <0.001
% of ID4+ cells
Control
BMP6
p <0.001
% of KI67+ cells
Control
BMP6
I
p <0.001
Cell number ratio relative to GFP virus
GFP virus
Id4 virus
p=0.012
% of EdU+ cells
GFP virus
Id4 virus

Fig 2': Regulation of ID4 in neural stem cells. A: IF for ID4 in spinal cord neural stem cells placed in the indicated conditions for EGF/FGF2 (EF). Cells were analyzed in the presence of growth factors (+) which were removed for 3 days (-) to induce differentiation and then added back for 3 days (+). **B**: WB (left panel) and QPCR (right panel) for ID4 in neural stem cells placed in the indicated conditions (n=3 independent experiments, test=one-way ANOVA and Tukey posttest). Numbers on the WB show relative quantification of ID4 signal normalized by actin. **C:** IF for ID4/GFAP and ID4/MAP2 in differentiated neural stem cells show that ID4 is preferentially detected in $GFAP^+$ not in $MAP2^+$ cells (white arrows). **D:** IF for p-ERK in differentiated neural stem cells infected with control (luciferase) or activated RAS lentivirus. Quantification is provided on the right panel (average % of cells +/- S.E.M, n=3 independent experiments, t=unpaired t-test). **E:** Left panel: Aspect of differentiated neural stem cells infected with control (luciferase) or RAS lentivirus. Round and probably mitotic cells are present (black arrow) with the activated RAS virus. Ki67 IF and quantification confirmed the presence of a high number of proliferative cells with the activated RAS virus (average % of cells +/- S.E.M, n=3 independent experiments, t=unpaired t-test). **F:** IF for ID4 in differentiated neural stem cells infected with control (luciferase) or activated RAS virus. Quantification is provided on the right panel (average % of cells +/- S.E.M, n=3 independent experiments, t=unpaired t-test). **G:** Images of NS derived from activated RAS virus infected cells and growing without growth factors (upper image). In the same condition, no NS were obtained from luciferase virus-infected cells (lower image). When placed in differentiation conditions with serum, RAS-infected NS generated few ID4+ cells and many Ki67+ cells compared to normal NS (middle panel). Right panel provides quantification for $ID4^+$ and $Ki67^+$ cells (average % of cells +/- S.E.M, n=3 independent experiments, t=unpaired t-test). **H:** IF for ID4 and Ki67 in growing neural stem cells treated with or without BMP6 10 ng/ml. Quantification is provided on the right panel (average % of cells +/- S.E.M, n=3 independent experiments, t=unpaired t-test). **I:** Effect of overexpression with control (GFP) or ID4 retrovirus on neural stem cell growth. (n=3 independent experiments, t=unpaired t-test). The upper diagram shows the cell number ratio +/- S.E.M obtained after 5 days in GFP or ID4 retrovirus-infected cells. The lower panel shows the % of EdU+ cells +/- S.E.M (3 hours EdU incorporation) obtained in the different conditions.

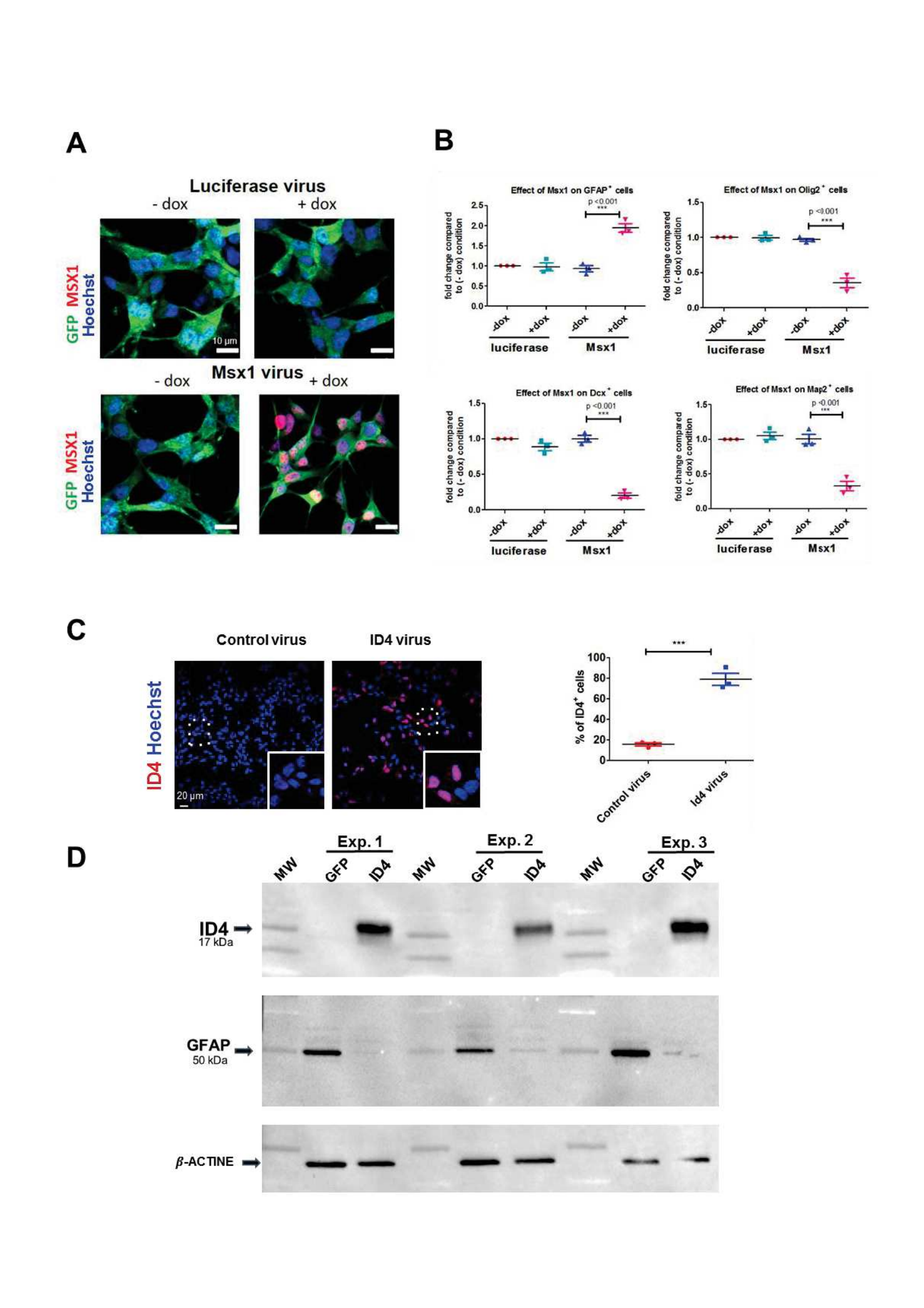

A
Luciferase virus
- dox
+ dox
GFP MSX1 Hoechst
10 µm
Msx1 virus
- dox
+ dox
GFP MSX1 Hoechst
B
Effect of Msx1 on GFAP+ cells
p <0.001
***
fold change compared to (- dox) condition
-dox
+dox
-dox
+dox
luciferase
Msx1
Effect of Msx1 on Olig2+ cells
p <0.001
***
fold change compared to (- dox) condition
luciferase
Msx1
Effect of Msx1 on Dcx+ cells
p <0.001
***
fold change compared to (- dox) condition
luciferase
Msx1
Effect of Msx1 on Map2+ cells
p <0.001
***
fold change compared to (- dox) condition
luciferase
Msx1
C
Control virus
ID4 virus
ID4 Hoechst
20 µm
***
% of ID4+ cells
Control virus
Id4 virus
D
Exp. 1
Exp. 2
Exp. 3
MW
GFP
ID4
ID4
17 kDa
GFAP
50 kDa
β-ACTINE

Fig S1': MSX1 and ID4 overexpression. **Related to Figure 7. A:** IF of MSX1 on cells infected with luciferase or MSX1 inducible-lentivirus, with or without doxycycline (dox). **B:** Effect of MSX1 overexpression on spinal cord stem cell differentiation (n=3 independent experiments, test=one-way ANOVA + Tukey post-test). Diagrams show the average of fold change +/- S.E.M of cells positive for the indicated marker. **C and D:** ID4 overexpression in growing neurospheres (passage 3) derived from the adult spinal cord. **C**: IF of ID4 on cells infected with control (GFP) or ID4 retrovirus. Quantification is provided on the right panel (average % of ID4+ cells +/- S.E.M, n=3 independent experiments, test=unpaired t test). **D:** WB performed on proteins extracted from cells infected with control (GFP) or ID4 virus showing increased ID4 but decreased GFAP. 3 independent experiments (Exp.) are shown. MW=molecular weights.

References

Barnabé-Heider, F., Göritz, C., Sabelström, H., Takebayashi, H., Pfrieger, F.W., Meletis, K., and Frisén, J. (2010). Origin of new glial cells in intact and injured adult spinal cord. Cell stem cell *7*, 470-482.

Becker, C.G., Becker, T., and Hugnot, J.-P. (2018). The spinal ependymal zone as a source of endogenous repair cells across vertebrates. Progress in neurobiology.

Hugnot, J.-P. (2013). Isolate and culture neural stem cells from the mouse adult spinal cord. In Neural Progenitor Cells (Springer), pp. 53-63.

Martynoga, B., Mateo, J.L., Zhou, B., Andersen, J., Achimastou, A., Urbán, N., van den Berg, D., Georgopoulou, D., Hadjur, S., and Wittbrodt, J. (2013). Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence. Genes & development *27*, 1769-1786.

Ramos, C., and Robert, B. (2005). msh/Msx gene family in neural development. Trends in Genetics *21*, 624-632.

Rosenberg, A.B., Roco, C.M., Muscat, R.A., Kuchina, A., Sample, P., Yao, Z., Graybuck, L.T., Peeler, Ruzinova, M.B., and Benezra, R. (2003). Id proteins in development, cell cycle and cancer. Trends in cell biology *13*, 410-418.

Sabourin, J.C., Ackema, K.B., Ohayon, D., Guichet, P.O., Perrin, F.E., Garces, A., Ripoll, C., Charité, J., Simonneau, L., and Kettenmann, H. (2009). A mesenchymal-like ZEB1+ niche harbors dorsal radial glial fibrillary acidic protein-positive stem cells in the spinal cord. Stem cells *27*, 2722-2733.

Perspectives

The first part of the project is an interesting shift in understanding the environment of the NSCs cell niche. Our study is the first to show transcriptomic, morphological and functional differences in the stem cell niche of human and mouse spinal cord. In this publication we identified the genetic profile of the mammalian (human and mouse) EZ, in which we identified the genes commonly and differentially expressed among both species. In addition, this study led us to identify various sub-population of cells that reside in the niche, focusing on the signaling pathways regulating their initiation and quiescence. Starting from this study we could go further to unravel other mysteries behind the human and mouse EZ niche.

Starting with differences identified in the ventral part of the EZ regarding the existence of CSF-contacting neurons. In fact we showed that CSF contacting neurons reside in the ventral part of the central canal and they express various specific markers such as DCN, PKD2L1 and TAL1 [1]. Similar profile was identified in the niche of regenerative models such as zebra fish and salamander [1], but those cell types remained almost unchanged in response to injury. An intriguing question is, what is the difference between CSF-Ns in regenerative and mouse models? And do they participate in the regeneration process after injury? To answer this question one could use transgenic mouse models targeting this specific population of cells and perform lineage study to follow their initiation. Following this, transcriptome analysis is necessary and to be more precise single cell analysis should be done, this allows to identify sub-populations within CSF-Ns and to identify the genetic signature of those cells. Comparing these data to already existing data base on CSF-N from regenerative models allow to understand what makes the differences among different species. Another interesting question is, why mammals do not regenerate after injury? And how CSF-Ns could be modulated in the context of regeneration? Interestingly similar comparative analysis is required that will lead to understand the function of CSF-Ns in injury context and how they differ from CSF-Ns in regenerative species. As these models showed an active regeneration by generating new neurons from the ventral part of the EZ niche. However CSF-N markers did not label the ventral part of the human EZ central canal, rather DCN for example is expressed in blood vessels [2]. One explanation is that this population of cells doesn't exist in the human, or it is labeled with other markers that to be identified. For that a detailed analysis using our data set and other data from the literature we might identify human genes that codes for the CSF-Ns markers.

In this publication we showed that CSF-Ns (DCN+/PKD2L1+/TAL1+) in the mouse ventral EZ expressed FOXA2, this population of cells were different from the ventral ARX+ cells. Interestingly in the human ventral EZ, FOXA2+ cells were also ARX+ indicating that there is at least two different population of cells that differ between mouse and human (figure S2 and

S4). Also this could point again on the absence of CSF-Ns in the human or there are other genes that encodes for other human specific CSF-Ns in the human. It will be interesting to target FOXA2+/ARX- cells in mouse EZ and the FOXA2+/ARX+ cells in human EZ and assess the differences in these two populations. This could be done using mouse transgenic models and single cell analysis to identify the genetic signature of both cell types and their characteristics, in which this analysis could lead to unravel the function of these two populations. One question could still under debate is whether the ARX+ cells found in human are derived from the floor plate as the mouse ARX+ cells? Or they have different origin?

Following this we identified another quiescent population of cells residing in the dorsal roof of the mouse and human EZ. This population of cells showed MSX1 expression co-localized with high ID4 expression under the regulation of BMP pathway. Moreover our *in-vitro* data showed that adding BMP6 caused increase in MSX1 and ID4 expressions and lead to decreased cell proliferation. In addition we identified a sub-population of MSX1+/ID4+ cells that are also positive to VEGFR3, in which this protein also labeled a population of ventral cells indicating dorsal-ventral regionalization. However it is interesting to follow the scenario *in-vivo*, and assess the influence of BMP6 and VEGR3 on the dorsal EZ cells in which it is lacking in our study. So what is the influence of induced high expression of BMP6 and/or VEGFc on the MSX1+/ID4+ cells and the dorsal ventral regionalization of EZ cells? To answer this question, two methods can be followed. The first is to inject the cytokines (BMP6 and VEGFc) directly in the spinal cord, then we can assess whether the in-vivo expression of MSX1 and ID4 expression is modified and whether the dorsal-ventral regionalization is modified. In this manner we might encounter some limitations, for example whether there is effect or not it will be difficult to identify whether it's due to endogenous or exogenous signals. The second way is through injection of BMP6-AVV and VEGFc-AVV into the lateral ventricle in the brain in which we could solve the problem raised by the first approach, so that we can follow and identify the exogenous cytokine we introduced. In addition to this it would be interesting to assess the activation of VEGFc on MSX1-Tomato+ cells *in-vitro*, a missing experiment in our study. This experiment can simply done by treating cell culture with VEGFc for few days and assess proliferation and differentiation. Such aims would allow us to identify the function of these pathways both *in-vitro* and *in-vivo,* and how they influence the protein expression profile within the EZ niche.

Focusing on the MSX1+ cell population it will be interesting to identify the RNA profile of this population in particular. In which we will launch a transcriptome analysis MSX1-tomato+ cells and control FOXJ1-GFP+ cells as FOXJ1 labels the EZ cells. The cells will be directly derived

from spinal cords of double transgenic mice MSX1-Tomato/FOXJ1-GFP. Such analysis will allow to identify several sub-populations within the MSX1 population evidenced by the presence of MSX1+/GFAP+/- and MSX1+/VEGFR3+/- in the **paper I**. An intriguing question is what could be the function of MSX1+ cells in intact and injured spinal cord? And could spinal cord injury activate quiescent MSX1+ cells in the dorsal roof of EZ? Performing acute SCI on MSX1-tomato mice would allow to answer both questions. We could follow activation, migration, and differentiation of MSX1-Tomato+ cells in the injured spinal cord. Also we could assess whether dorsal-ventral regionalization in the EZ is altered on not. In addition to this we can go so far by assess function of MSX1+ cells in context of degenerative diseases such as ALS and SMA, either through generating double transgenic by crossing MSX1-Tomato mouse with degenerative mouse model, or through transplantation of MSX1+ cells in injured spinal cord or in the spinal cord of degenerative model. Thus we can assess integration of these cells in the treated spinal cord and check for functional recovery through behavioral tests. Finally, we noticed in our study that the human EZ is all labeled with MSX1 with high expression in the dorsal roof. It will be interesting to investigate the different expression of MSX1 in mouse and human EZ. So whether this population of endougenous NSCs could could be a target for therapeutic purposes for SCI and degenerative diseases is still a debatable issue to be investigated.

Part II

Spinal cord injury activates the central canal stem cell niche

Project 2 (in preparation)

Spinal cord stem cell niche: from quiescence to activation after spinal cord injury.

Spinal cord stem cell niche: from quiescence to activation after spinal cord injury.

H Ghazale[1], C Chalfouh[3], C Ripoll[1], N Guerout[3], JP Hugnot[1,2]

Author affiliations

1: INSERM U1051, INM, Hopital Saint Eloi, 80 avenue Augustin Fliche, 34091 Montpellier, France

2: University of Montpellier, Faculté des Sciences, Place Eugène Bataillon, 34095 Montpellier cedex 5, France

3: Institute for Research and Innovation in Biomedicine (IRIB), 76000 Rouen, France

Keywords: SCI, NSCs, activation, gene expression.

Abstract

The spinal cord central canal harbors the ependymal zone (EZ), considered as the neural stem cell niche. Unlike the regenerative EZ in invertebrates, the adult EZ in mammals is quiescent. Spinal cord injury (SCI) is an event leading to neuron degeneration, cytotoxicity and functional deficits. On the other hand SCI is a leading cause of NSC niche activation. Our study showed the RNA profile differences of the EZ in intact and injured spinal cord, highlighting the main molecular pathways involved in the EZ activation. We identified the STAT3 signaling in the injured spinal cord that could be coupled with the activation of oncostatin (OSM) and its receptor (OSR) and may lead to hyper-activation of astrogliosis through driving GFAP, THBS2 and CRYM expression. However there is a de-activation of the Hippo pathway characterized by the turn off of YAP/TAZ-TEAD transcription machinery. Finally our data showed upregulation of the transcription factor (TF) Olig1 in the EZ a characteristic of motoneuron and oligodendrocyte progenitor cells specification. These findings demonstrate that, the quiescent adult EZ zone is reactivated following injury through the activation of signaling molecules involved in astrogliosis and remodeling of the EZ through developmental TFs over-expression.

Introduction

Spinal cord injury or neurodegenerative diseases are major causes of neuron loss, in which these neurons are not replaced in mammals [3]. Following an injury, spinal cord faces the formation of inhibitory glial environment, intensive demyelination and axon degeneration, and neural cell death. Combined together these factors inhibit the complete regeneration and functional recovery after injury [4]. Although these factors such as glial scar formation played a role against the self-repair mechanism in the first hours after injury, novel studies showed that astrocytes and ependymal cells might have pro-regenerative effect [5], [6]. So therapeutically, endogenous ependymal cells could be an alternative method targeted to treat SCI, or the other way round SCI could activate the quiescent state of the stem cell niche [7], [8].

The adult mammalian stem cell niche in the spinal cord is relatively quiescent. The mammalian ependymal zone (EZ) is a pseudo-epithelium (Express β-catenin and CD24) originating mainly from the ventral part of the developing neuroepithelium. This niche is characterized by the existence of various cellular populations expressing specific set of transcription factors (as shown in the first publication). Talking about lower vertebrates it's well-known that SCI activate the neurogenic niche and derive the proliferation of precursor/stem cells, and they contribute to the regeneration mechanism [9]. On the other hand, the contribution of these cells into the regeneration process in rodents is still controversial, while in human it is almost absent [10].

On behalf of the first publication, the RNA profile of the EZ after injury is still lacking. And since one clinical interest is to address the effect of SCI on the endogenous stem cell activation. This study is providing a detailed RNA profiling comparing intact spinal cord EZ Vs. injured spinal cord EZ in mouse. This study allows to identify the key genes inducing the activation of the quiescent NSCs. As well as could unravel the role of key endogenous NSC in the context of repair after injury. This study could be the first to undertake a comparative description of the importance of cells in the ependymal zone between regenerating and non-regenerating vertebrate species.

Materials and Methods

1. Animals

Mice were handled following the guidelines of the Animal Care and Use Committee of the National Institute of Health and Medical Research (INSERM) who approved this study in accordance with the European Council directive (2010/63/UE) for the protection and use of vertebrate animals. Adult CD1 mice (3 months, Charles River, France) were used for micro-dissection, RNA profiling and comparison between non-injured vs. injured spinal cord. C57BL6 wild type mice (3 months) were used for histological analysis. Msx1-CreERT2/Rosa-Loxed Tomato transgenic line [11] was obtained from Y Lallemand (Pasteur Institute, Paris). To induce recombination in Msx1-CreERT2 animals, 100 µl of tamoxifen (Sigma, T5648, 20 mg/ml dissolved in corn oil) was injected intraperitoneally for 4-5 days. These mice were used for spinal cord injury, and histological analysis.

2. Spinal cord Injury

Adult CD1 mice (3 months old) were anesthetized after inhalation of 1.0%–1.5% isoflurane gas; following vertebral thoracic 9 level (T9) laminectomy a lateral spinal cord hemisection (HS) was performed under microscope using a micro knife (10315–12, fine science tools (FST)), as described previously [12]. Lesions were done at T9 level to obtain monoplegia. Both muscles and skin over-laying the lesion area were sutured, and animals remained under visual monitoring during 2 h over the recovery period before returning them to their home cages. 4 mice were used to micro-dissect the central canal for the RNA profiling that is performed after 48 hours following injury. Same laminectomy was followed on 3 mice for histological studies that are dissected 48 hours following injury.

3. Tissue micro-dissection

Same procedure was followed as described in the first publication. Briefly, mice spinal cords (injured and non-injured) were flash frozen in N2 without chemical fixation. Frozen sections (30 µm thick, **T9-T10** thoracic part **for mouse**) were obtained at –23°C to prevent RNA degradation using a CM3050S microtome (Leica Microsystems, Wetzlar, Germany) and were mounted on PEN-membrane 1 mm glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) that had been pretreated to inactivate RNase. Immediately following dehydration, laser microdissection was performed using a PALM MicroBeam microdissection system version 4.6 equipped with a P.A.L.M. RoboSoftware (P.A.L.M. Microlaser

Technologies AG, Bernried, Germany). To limit RNA degradation, samples were collected for up to 15 minutes per slide and microdissected tissue was lysed with 250 µl of lysis buffer (Promega, Madison, USA). The samples were stored at −80°C until extraction was performed using the ReliaPrep RNA cell Miniprep System (Promega, Madison, USA) according to the manufacturer's protocol and eluted with 14 µl of RNAse-free water.

4. RNA profiling and bioinformatic analysis

Same procedure was followed as described in the first publication. Briefly, RNA profiling was performed using Affymetrix microarray technology. Hybridization targets were obtained following a double amplification procedure according to the protocol developed by Affymetrix (GeneChipTwo-Cycle Eukaryotic Target Labeling Assay; Affymetrix, USA). A hybridization mixture containing 10 µg of biotinylated cRNA was generated. The biotinylated cRNA was hybridized to HT_HG-U133_Plus_PM (human) and HT_MG-430_PM (mouse) Affymetrix microarrays. Four mice samples of each condition (EZ) were analyzed in total. The microarrays were scanned using the Affymetrix Gene Atlas scanner. Gene lists were analyzed with DAVID Bioinformatics Resources 6.8 for gene enrichment analysis. Data are available at the functional genomics data Gene Expression Omnibus (GEO) repository (series record GSE118445).

5. Mouse histology

Same procedure was followed as described in the first publication. Briefly, Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused intracardially with 10 ml of phosphate-buffered saline (PBS) followed by 50 ml of 4% formaldehyde-PBS solution (pH 7.0). After dissection spinal cords were post-fixed in the same solution for one hour and cryopreserved by successive immersion in 10, 20, and 30% sucrose solutions in PBS for at least 6 h. Cervical, thoracic and lumbar parts of the spinal cord were cut, embedded in OCT medium, rapidly frozen in liquid N2-cooled isopentane and cryosectioned (14µm) (Leica apparatus).

Immunofluorescences (IF) were performed with primary antibodies listed below (supplementary table 1) on sections permeabilized for one hour with 0.1% Triton 100x and 5% donkey serum. Secondary antibodies (Alexa488 or Alexa594-conjugated species-specific anti mouse, rabbit or goat) were purchased from the Jackson Laboratories. Incubations without primary antibody or with antibody recognizing antigens not present in the sections (monoclonal anti DYKDDDDK Tag) were used as negative controls. Nuclei (blue in all images) were stained with Hoechst 1 µg/ml for 10 min. The quality of staining was evaluated by two independent

investigators (HG, and JPH). Images were taken using optical sectioning with structured illumination (Zeiss apotome microscope).

6. Statistical analysis

All experiments and stainings were performed at least twice and most of them were done three times. Significances: *** ($p<0.001$), ** ($p<0.01$), * ($p\leq0.05$)

Results

Genes enriched in the intact mouse spinal cord vs. injured spinal cord.

As previously described in the first paper, the RNA profile of human and mouse EZ has been described and identified (refer to paper I). Similar study has been launched to identify the RNA profile of mouse spinal cord EZ in intact and injured states. Such RNA profiling study will allow to identify the differential gene expression in quiescent and activation state of the EZ. The EZ of intact and pinched spinal cord was micro-dissected (figure 1A), and microarrays were performed on both RNAs. Heat map of the four samples indicated adequate clustering of the EZ in each condition (figure 1B). Volcano plot showed the up-regulation of 875 genes and down regulation of 606 genes after spinal cord injury with respect to control (fold change ≥ 2) (figure 1C). Some of the identified genes on the volcano plot were represented in a table, in which genes marked in red were also checked at the protein level to confirm the transcriptome data (figure 1D). The volcano plot data only shows some of the genes that are significantly up and down regulated. Gene ontology revealed the molecular functional mechanisms of the up and down-regulated genes (figure 1E, F). Interestingly, genes involved in cell adhesion were also up-regulated such as extracellular matrix protein 1 (ECM1) and Fibronectin. Moreover, genes involved in mitotic nuclear division were also up-regulation, an indication of enhanced activation and proliferation of EZ cells. Of these genes, Ccnd1, encoding cyclin D1 is a major candidate involved in mitotic division (supplementary excel file). On the other hand, a massive down-regulation was observed in the genes involved in cilium movement (figure1 F). The decrease in cilium movement is not an indication of decreased Foxj1 expression, a marker of ciliogenesis. Rather it could influence the response of cilia to flow and receiving external signals, and disrupt the regulation of signal transduction [13].

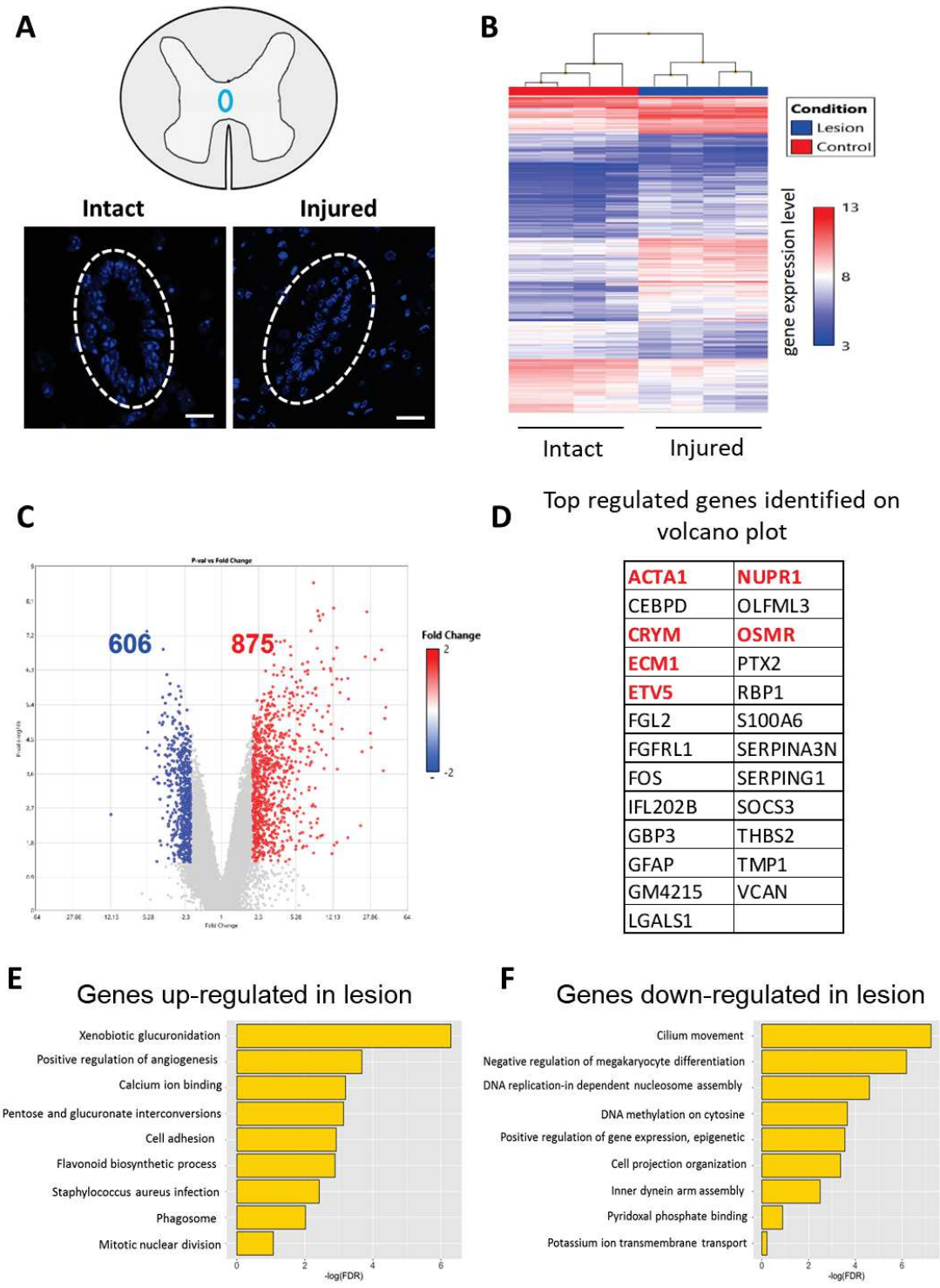


Figure 1: RNA profiles of mouse EZ following SCI. (A) Schema showing the procedure of lase-micro dissection of intact and injured spinal cord EZ. Microdissected EZ region is delimited with dotted circles. Scale bars = 100 µm. **(B)** heat map of hierarchical clustering of genes expressed in EZ region in the 4 control mice and 4 injured mice samples. **(C)** Volcano plot showing the up-regulated and down-regulated genes (606 and 875 respectively) with a fold change ≥ 2. **(D)** Summary of the most regulated genes. **(E, F)** gene ontology showing up and down-regulated genes and their corresponding functional mechanisms after SCI.

Validation of the up-regulated genes by Immunofluorescence

Following RNA profiling of the mouse EZ, we confirmed the up and down-regulation of some genes at the protein level. Mice were subjected to the same injury as the mice used for RNA profiling. Immunostaining revealed decreased ACTA1 (Actin alpha 1) expression in the EZ after injury, with a staining restricted to few cells in the parenchyma (supplementary figure 1). It is a conserved protein of the actin family that play a role in cell motility, structure and integrity. Among its related pathways are Integrin Pathway and SMAD Signaling Network [14]. Gene Ontology (GO) annotations related to this gene include structural constituent of cytoskeleton and myosin binding. On the other hand there were an up-regulation in CRYM (Mu-Crystallin), almost exclusive to the ependymal zone (figure 2). Fibronectin showed increase in the EZ as well as in the parenchyma, while ECM1 (extracellular matrix) increase was major in the parenchyma rather than the EZ (supplementary figure 1 on page 181). This increase could help the rearrangement and reorganization of the spinal cord environment after injury, and could be interpreted by the increase in cell adhesion (figure 1E). A remarkable up-regulation of Gas1 and Netrin genes after SCI, which has been confirmed by immunofluorescence. The EZ of injured spinal cord is highly positive for GAS1 and NETRIN compared to control (figure 2). GAS1 is induced by Wnt signaling is required for proliferation of progenitors of the cerebellar granule cells and Bergmann glia [15], while NETRIN could be implicated in axon guidance in response to injury [16]. The EZ was immunonegative for ETV5 in the control spinal cord, but interestingly there was a massive increase in the ETV5 nuclear expression following SCI (figure 2). This increase could be related to the increase in GFAP, and Thbs2 (figure 1D) as ETV5 corresponds to the ETs family that play a role in perinatal gliogenesis mainly astrogenesis [17], [18]. This was coupled with an activation of another astrogenesis inducer [17], P-STAT3. Remarkably the control EZ was expressing cytoplasmic p-STAT3, and upon injury it is activated and translocated to the nucleus. Combined together those two TFs may induce astrocytes from the EZ. The phosphorylation and activation of STAT3 can be induced in part by the increased expression of OSMR after injury (figure 2). Some data showed that OSMR increase after inflammation enhancing the activation of STAT3 [19], on the other hand STAT3 could be required for GFAP-OSMR clustering to induce astrocyte differentiation [20]. OSMR is activated by oncostatin (OSM) which is also increased after injury (supplementary figure 1). Although RNA profiling data showed almost no change of OSM expression in the EZ after injury, but immunostaining showed increased OSM expression in the EZ and parenchyma. Moreover, our RNA profile data showed a significant up regulation of the phospho-extracellular signal-regulated kinase (p-ERK). This data was

confirmed by p-ERK immunostaining that increased in the EZ after injury (figure 2). Interestingly, p-ERK is a protein known to play a role in cell division, growth, and proliferation. One of the identified pathways that could play a key role in the activation and proliferation of EZ cells, is the hippo pathway that involves YAP/TAZ, and TEAD transcription factors. Immunostaining showed similar expression of both YAP (supplementary figure 1), and YAP/TAZ (figure 2) in control and injured spinal cord, while TEAD expression is decreased in the EZ of the injured spinal cord, and its cytoplasmic expression is restricted to few cells in the parenchyma (figure 2). To the knowledge, activated YAP, and YAP/TAZ enter the nucleus, dimerize and couple with DNA binding TEAD protein to activate gene expression [21]. How the decrease of TEAD1 protein could influence the transcriptional machinery after injury is still unclear.

Another striking results observed both in RNA data and immunostainings is, the significant increase of Olig1 TF in the EZ following injury (figure 1 & 2). One to two cells were observed either dorsally or ventrally in the EZ, however not all sections showed Olig1+ cells. During development Olig1 is expressed mainly ventrally in the EZ, where those Olig1+ cells give rise to motor neurons. The EZ expression of Olig1 decrease in the early postnatal stages to disappear late in the adult stage when neuron specification has occurred [21]. The re-expression of Olig1 in the EZ of injured spinal cord is promising for active repair mechanism.

Combined together these data showed activation of the extracellular matrix responsible proteins, coupled with enhancement of pathways required for cell activation, proliferation and gliogenesis, with Olig1 a promising target for injury repair.

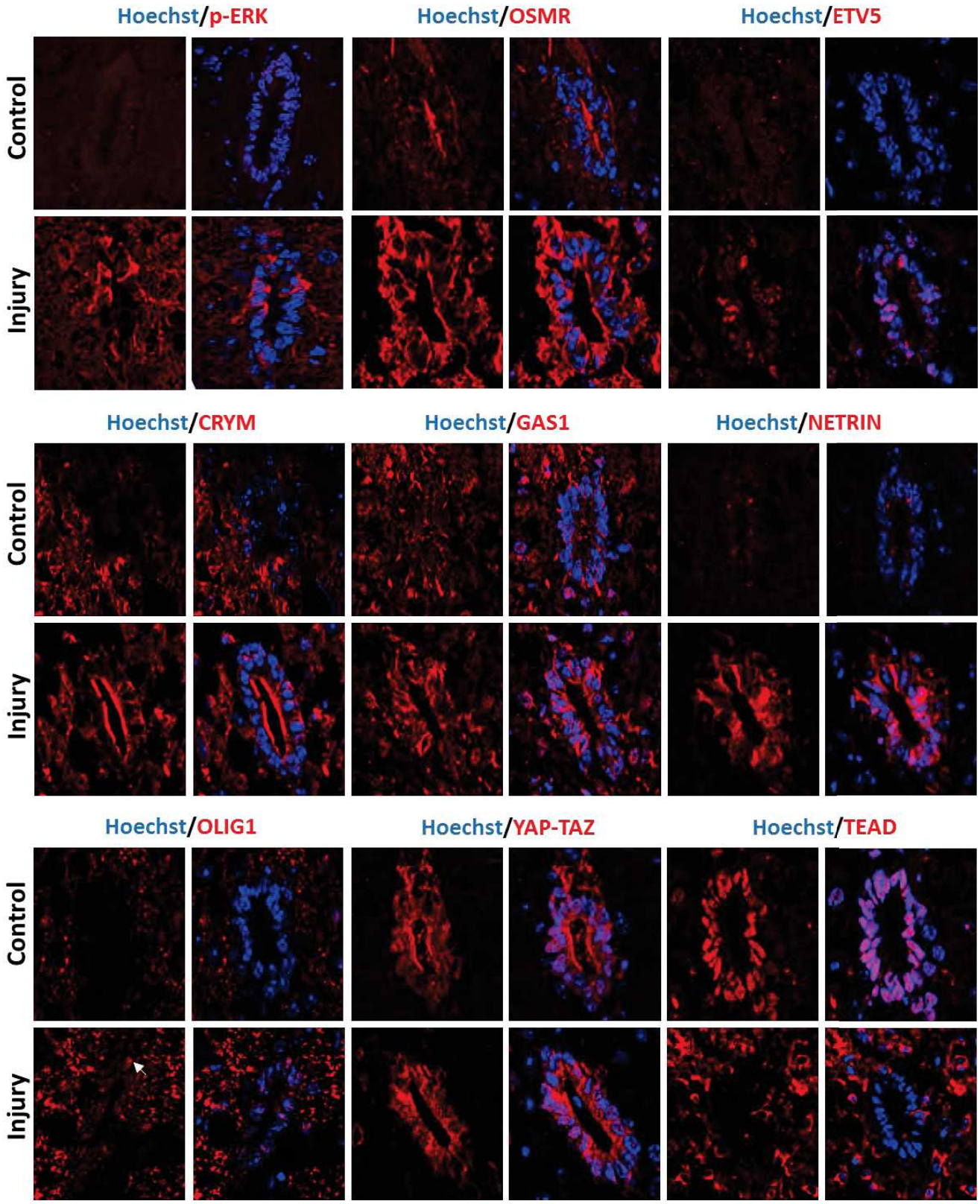


Figure2: EZ characterization of intact vs. injured spinal cord. IF for the indicated proteins in the adult mouse EZ (thoracic level). Images are oriented with ventral part to the bottom. The white arrow on OLIG1 staining show positive cell in the dorsal part. Presented images are representative of 6 sections per animal, n=2 mice analyzed. Scale bar (shown on first image) = 20 µm, and Hoechst is used as a nuclear stain in blue.

Discussion

In this study RNA profiling was used to explore the adult mouse EZ in control and injured spinal cord. We built a new corpus of knowledge on this particular CNS region following an injury. Our preliminary data revealed important and new characteristics of the activated stem cell niche following an injury compared to the quiescent niche. Our data revealed an up-regulation of genes implicated in increased proliferation, adhesion, axon guidance and astrogenesis. Thus the EZ stem cell niche is an active zone and a territory of endogenous stem cells that are implicated in the repair mechanism.

Following a spinal cord injury, there is a massive destruction of the neuronal function in the spinal cord [22]. It is caused by axonal damage, dysfunction, and degeneration, and this damage is irreversible [22], [23]. CRYM is known to be expressed in a wide range of tissues, including brain where it is expressed in the descending projection neurons of the motor cortex [24]. While in the spinal cord it is expressed in descending corticospinal tracts axons that are localized dorsally and ventrally in the grey and white matter [23]. To the knowledge, Pawar et al showed that CRYM is co-localized with NF-200, an axonal marker, in which those double positive fibers appeared to cross the injured area and reenter the adjacent spared parenchyma. Thus, CRYM expression is increased after injury and support the regeneration of descending axons, where its increase in the EZ (figure 1 & 2) following injury requires a further investigation. It could be possible that CRYM is involved in driving stem cells to enhance axonal regeneration and guidance as well as it could be possible that it enhance the inflammatory response through macrophages as a minority of cells are co-labeled with F4/80 a positive marker of macrophages [24]. In addition to this single cell analysis on the V-SVZ NSCs in the brain revealed the existence of 5 sub-populations of astrocytes, in which astrocyte type-2 are enriched in the expression of CRYM. It appeared that astrocyte type-1 cells are involved in deriving activation of quiescent stem cells into transit amplifying cells to generate OB neurons. Also they showed that this lineage is coupled with the generation of OPCs during the activation process [25]. So the increase of CRYM particularly in the EZ following injury could be an indication of the presence of astrocyte type-2 cells that are undergoing activation.

One of the major events that follow the SCI is the morphological changes in the intermediate filament content and extracellular matrix material that may push EZ cells to undergo epithelial-mesenchymal transition [26]. Interestingly we showed an increase of the adhesive glycoprotein fibronectin in the EZ as well as a significant increase of ECM1 protein in the parenchyma (figure 1 & 2), this could indicate a repair mechanism is ongoing following SCI, as EZ cells are

undergoing EMT and a rearrangement procedure, and it is a major indication of ependymal outgrowth, and axon regeneration. Moreover those two mentioned proteins could be localized with vimentin and GFAP an indication of endogenous stem cell changes as well as astrogliosis [26]–[28]. Another protein involved in neurite outgrowth and axon guidance is NETRIN1, where it showed an up-regulation in the EZ after injury (figure 1 & 2). NETRIN1 are secreted proteins expressed in various cell types during development and in subset of neurons, where coupling with its receptors play a role in axon attraction or repulsion [29], [30]. Genetic mutation to this protein causes defects in motor neuron axon projections, and this protein receptor coupling is known to be highly affected in Huntington disease, in which NETRIN1 can be a target for treating axon degeneration and guidance [16], [31]. However its expression in EZ stem cells is still not well illustrated, and further investigation is required to understand the overexpression of NETRIN in the EZ following an injury.Whether the ependymal cells are the source of secreting this protein? Or is it the target of NETRIN binding to its receptor? Questions have to be addressed to identify the role of netrin in stem cell activation.

Of the identified genes, are those regulating cell fates during development. GAS1 was identified as a negative regulator of the SHH signaling in various tissues [32] while recent data showed that GAS1 is an agonist of the SHH signaling [33], [34]. In which GAS1 mutants showed phenotypic defects due to decreased shh. As ventral neural development is regulated by shh signaling, GAS1 could be a major regulator of the ventral cell fate identity and patterning. The scenario starts at the ventral midline with the specification of floor plate cells that require the highest level of Shh signaling [35]. The scenario involves a localized expression and activation of FoxA2, a direct transcriptional regulator of Shh [15]. Importantly, despite the strong expression of Gas1 in dorsal domains, GAS1 mutants showed no changes in the specification of general dorsal cell identities (Pax6+, Pax7+) and specific Msx1+ roof plate and Math1+ dp1 progenitors. Thus Gas1 functioning to specifically modulate the level of Shh signal that cells are exposed to during neural tube patterning [34], [35]. However the increase of GAS1 in the EZ after injury was not ventrally restricted, rather its expression appeared dorsally and ventrally (figure 2). And this could be also explained by the fact that GAS1 is induced by Wnt signaling that controls the dorsal patterning. And it is proven that Wnt induce GAS1 expression that is required for proliferation of progenitors of the cerebellar granule cells and Bergmann glia [15], [33]. We can conclude from our data and from the literature that, SCI injury enhance both Wnt and Shh signaling that influence dorsal and ventral expression of GAS1 which regain the embryonic feature patterning and enhance proliferation in the EZ.

Staying in the scenario of patterning during embryonic development, one gene family that could fit is the Gli family, which is also regulated by the Shh signaling. As described previously, the most ventral cell type in the neural tube, requires higher levels and longer durations of Shh signaling than p3 cells. Consistent with this, mice lacking the trans-membrane protein Smo or Gli transcription factors, which are involved in the transduction of Shh signal, demonstrated that FP specification requires Shh signaling [36], [37]. Gli3 which belongs to the Gli family is known to be a positive contributor to shh signaling and act with GAS1 in patterning, this could explain the increase of its expression after SCI (supplementary figure 1). Gli3 along with Gli2 are able to activate shh signaling, in which loss of Gli3 function results in reduced shh. Although Gli proteins are not essential to derive all motor neurons and V0-V2 interneurons, but they are essential to regulate normal motor neurons differentiation and the normal patterning of ventral cell types [38], [39]. Gli3 mutant embryos showed additional phenotype resulted in massive proliferation in the ventral most part of the spinal cord. This was tested by the incorporation of BrdU that increased in the ventral part including ventral midline with the loss of Gli function. The increase of BrdU is concomitant with the increase of cyclin D1, a G1 cyclin and a target of shh which indicates the over-proliferation is due to the disruption of the cell cycle [39], [40]. In addition to the involvement in the spinal cord patterning, Gli3 could be involved in controlling cell fate and adhesion to establish a proper neurogenic niche [41]. Shh signal plays a critical role in maintaining the proliferation of NSCs and other progenitor cells in adults [42]. However Gli3 is expressed by RGCs prior to shh in embryonic stage, so one could raise a question whether Gli3 in the SVZ plays a role in the development of the neurogenic niche in the absence shh signal?

Expression study showed that Gli3 is expressed in the NSCs and ependymal cells of the SVZ, while Gli1 (shh signaling) was so restricted embryonically and not involved in OB neurogenesis until E18.5. Instead Gli1 is expressed in cells capable of proliferation in the SVZ. For that Gli3 act as a repressor in RGCs during development and postnatal ependymal cells [43]. Conditional removal of Gli3 in RGCs before shh activation but after embryonic patterning is established, showed abnormal organization of the neurogenic niche [41]. Normally in the developing neurogenic niche, NSCs (GFAP+) and ependymal cells (β-catenin+) are arranged in a pinwheel structure at the lateral wall of the lateral ventricles [44]. In contrast to this, in the absence of Gli3 there was no apparent pinwheel structure with an up-regulation of GFAP in RGCs suggesting a delay in the niche maturation. On the other hand, it showed up-regulation of γ tubulin+ ciliary bodies suggesting mature ependymal cells with persistence of GFAP over-expression. So GFAP played a role in the distinction of NSCs and ependymal cells that failed

to develop in the absence of Gli3 [45]. The malformation of the neurogenic niche observed at P21 could also be due to the delay in ependymal cells maturation and the lack of proper specification of cell identity. This was clear by the decrease of Foxj1, a marker of mature ependymal cells, in the absence of Gli3 function, as well as due to the presence of Foxj1+GFAP+ cells in the Gli3 mutant embryos but not in the control ones [45]. Thus, Gli3 mutant cells with double identity are no more functional and are not proliferative in which they lost the NSCs characteristics, causing structural defects in the neurogenic architecture and leading to severe diseases. This confirms the importance of Gli3 function in maintaining niche structure and cell fate, a reason why it increased slightly after SCI (figure 2).

One could raise a question about the downstream events leading to the ectopic expression of GFAP in Gli3 mutant SVZ? So analyzing gene expression for identified genes that are involved in Shh and/or Notch pathways and contain Gli3 binding sites, could answer the question [46]. Such analysis is carried on E16.5 forebrain tissue from wild type embryos with that from Gli3 null mutant animals, a time point in which active Shh signaling is absent and any phenotypes observed is attributed to the loss of Gli3 function. Surprisingly, one of the dramatically changed genes was il6st that encodes a protein named gp130, a co-receptor subunit shared by the IL-6 family of cytokines including CNTF, OSM, IL-6, LIF and CT-1 [47], [48]. JAK-STAT molecules are downstream effectors activated by gp130 and the cytokine-specific receptors that transduce cytokine signals. Gp130 dimerization leads to phosphorylation of cytoplasmic tyrosine residues of the chimeric receptor protein, subsequent STAT3 and ERK1/2 phosphorylation, and transcriptional activation of gp130 target genes [49] . In E16.5 forebrain of Gli3 mutant both il6st and gp130 protein levels were increased, however, the expression levels of all the cytokine-specific co-receptors were unchanged [45], [47], [48]. Interestingly in our study we showed a significant increase of OSM and its receptor as well as a slight increase in the STAT3, in which p-STAT3 translocated from the cytoplasm to the nucleus after SCI (figures 1 & 2). Gp130 promotes GFAP expression and glial cell fate through the phosphorylation of the STAT3 transcription factor where a transient activation of STAT3 in a small population of SVZ cells at P4 is observed [50], [51]. To the knowledge, majority of p-STAT3+ cells also expressed GFAP, confirming that, increased gp130 level induce ectopic activation of STAT3 to induce GFAP overexpression. So it's clear that pSTAT3 directly activates GFAP gene transcription by binding to its promoter sequence [45], [52]. Focusing on the STAT3, it is a pathway widely addressed in the context of regeneration and CNS injury. Xenopus laevis tadpoles are characterized to have two developmental stages, pre-metamorphic and metamorphic Xenopus stages. It's know that xenopus laevis tadpoles can regenerate a

spinal cord after injury but this capability is lost during metamorphosis [53], [54]. Interestingly, these two developmental stages could allow to understand the molecular mechanisms of spinal cord regeneration. JAK-STAT pathway is one of the signaling pathways known to control several cellular responses after SCI in mammals. This pathway is involved in the transduction of several cytokines and growth factors such as OSM and its receptor. Activation of the JAK-STAT pathway leads to the phosphorylation of STAT3, which causes its translocation to the nucleus to bind DNA regulatory sequences and regulate gene expression [49], [55]. Following SCI different components of the JAK-STAT pathway have been used to characterize pathway activation such as an increase in the levels of cytokines [56] or phosphorylated STAT3 (figure 1 & S1) [57], as well as the increase of direct targets of this pathway, such as SOCS3 [58]. In addition to this, the activation of STAT3 is necessary for astrocyte involvement in the glial scar formation, which in turn activate the inflammatory response and support axon regeneration [59], [60]. Moreover gain of JAK-STAT function proved to be implicated in axon regeneration and collateral sprouting, enhancing motor recovery [61]. Following the increase of pSTAT3 in the EZ (figure 1 & S1), Sox2+ ependymal cells from the VZ proliferate after SCI and are necessary for spinal cord regeneration [54]. Taken together STAT3 co-localized with Sox2+ cells in the VZ, and there is stage-dependent differences in JAK-STAT activation in relevant cell types for spinal cord regeneration [54], [62]. Finally p-STAT3 increased expression after a SCI could play a critical role in promoting corticospinal remodeling and functional recovery [63].

Previous studies on brain ischemia and TBI showed that, STAT3 activation mediates astrocytes phenotypic changes that are characterized by cytoplasmic enlargement, elongation of their processes, and up-regulation of GFAP; this change is termed reactive astrocytosis. The GFAP upregulation is coupled with the upregulation of oncostatin (OSM), a major component of the STAT3 pathway [64]. Similar data were obtained in our study after the translocation of STAT3 into the nucleus, where OSM and OSMR are upregulated in the EZ following injury compared to the control (figure 1, 2, & S1). OSM belongs to the IL-6 cytokine family and signals using the gp130 receptor. OSM and OSR significantly increased in the EZ and parenchyma after SCI (figure 1, 2, & S1). Where OSM could mediate and provide neuroprotective effect against cytotoxic injury and promotes repair in demyelinated regions after injury [65]. Also it has been shown that OSM promotes activation of STAT3 selectively in Muller cells in the retina, which promotes protection of photoreceptors in a mouse model of retinal degeneration, suggesting that OSM may serve to induce glial-neuronal protective effects in other injury models [66]. So in our model the up-regulation of OSM and OSR may promote neuronal survival and

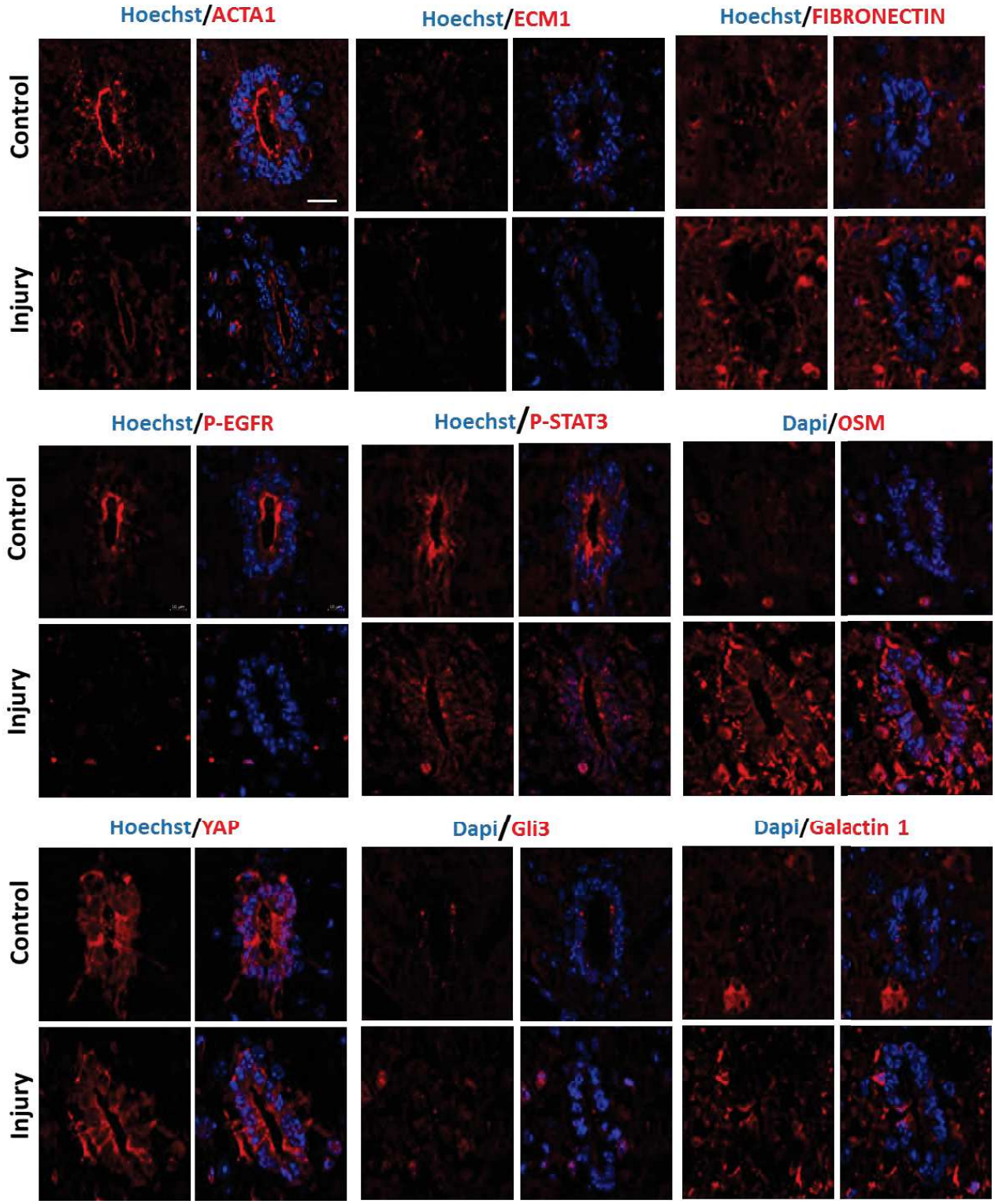
regeneration with evidence of decreasing lesion size, may be necessary to allow for the activation of neuroprotective pathways and inhibit the deleterious effects associated with reactive astrocytosis. and may serve a novel neuroprotective function and may be a new therapeutic target to enhance neurite growth and functional recovery [67], [68].

Another pathway appeared to be involved in the molecular changes and proliferation of the EZ following SCI is the Hippo pathway. It is a conserved pathway that regulates organ size and tumorigenesis through negatively regulating the oncogenic transcriptional co-activators yes-associated protein (YAP) and TAZ (WWTR1) [69]–[71]. Following activation, the Hippo pathway phosphorylates YAP, in which p-YAP undergoes subsequent degradation. On the other hand, when de-phosphorylated it enters to the nucleus after dimerizaton with TAZ, and interact with DNA binding protein family TEAD. Thus deriving transcriptional gene machinery that are involved in regulating various cellular processes, including cell survival, proliferation and differentiation [72]–[74]. However the function of YAP, YAP/TAZ, and TEAD in multiple organ development has been well described, while their function in the CNS development and injury still not yet clear. In our study we observed almost no change in YAP, Yap/TAZ expression in SCI compared to the control, but strikingly the nuclear expression of TEAD in the EZ of control SC decreased and almost disappeared from the EZ of the injured SC (figure 2 & S1). These data should be further analyzed by assessing the function of YAP in-vitro to understand its role in the context of EZ following SCI. In a normal situation, YAP is involved in the BMP suppressed differentiation of embryonic NSCs through a direct interaction with Smad1. BMP2 and other growth factors are highly expressed in mouse NSCs, suggesting a vital role of this family in maintaining NSC quiescence in the adult CNS [75], [76]. Various in vitro studies confirmed that BMP2 activation influenced self-renewal and proliferation of mouse embryonic NSCs through the neurosphere assay. Cultures were treated with BMP2 showed fewer neurospheres with smaller sizes, suggesting an inhibitory effect of BMP2 on self-renewal and proliferation of NSCs [77]. We obtained similar data when treated cultures with BMP4 through upregulating MSX1 and ID4 (refer to the first paper). YAP is the target of BMP2 signaling, in which activation of BMP2 signaling represses the YAP/TEAD transcriptional machinery in embryonic NSCs. These data suggests that BMP2 signal reduces the YAP/TEAD coupling and enhance the YAP/Smad1-4 interaction to maintain NSCs in their quiescent state or promotes neocortical astrocytic differentiation [78]. In fact this activity is reversed by noggin treatment a BMP antagonist [77], [79]. Moreover, in the developing brain, YAP was selectively expressed in NSCs and astrocytes [21], in which its deletion resulted in reactive astrogliosis. This drives microglial activation that is associated with decreased BBB function. Thus YAP in

astrocytes counter-act the hyper activity of the JAK/STAT3 inflammatory signaling through the induction of suppressor of cytokine signaling (SOCS) family gene expression [80]. This could explain our observation in which STAT3 pathway is activated with the up regulation of OSM and OSMR and the inhibition of YAP/TAZ through the decrease of TEAD1 in the EZ following SCI (figure 1, 2, & S1).

Of the major results we obtained following SCI is the presence of Olig1 cells in the EZ (figure 2). Normally intact adult EZ is devoted of Olig1, as its expression in the EZ is restricted to the developmental stage [81]. Evidence showed that Olig1 play essential roles in oligodendrocyte progenitor cell specification and differentiation in the developing embryonic spinal cord and brain. Also there is an evidence about Olig1 over-expression following SCI in the white and gray matter, but nothing is mentioned about EZ stem cell niche [82], [83]. During development Olig1 not only essential for oligodendrocytes but also essential for motoneurons from the ventral EZ and a subset of astrocytes and ependymal cells [84]. So our data demonstrates that the EZ regained the developmental phenotypes by expressing Olig1 following SCI. This might enhance the generation of motoneurons, oligodendrocytes, astrocytes, and maintaining ependymal cells in the SVZ. Thus Olig1 could be a therapeutic target to control endogenous stem cells in the EZ following injury. This study pave the way to identify vital molecular event involved in the use of endogenous stem cells as a therapeutic target for degenerative diseases and CNS injury.

Supplementary



Supplementary figure1 (S1): EZ characterization of intact vs. injured spinal cord. IF for the indicated proteins in the adult mouse EZ (thoracic level). Images are oriented with ventral part to the bottom. The white arrow on OLIG1 staining show positive cell in the dorsal part. Presented images are representative of 6 sections per animal, n=2 mice analyzed. Scale bar (shown on first image) = 20 µm, and Hoechst is used as a nuclear stain in blue.

Supplementary table 1: List of antibodies used for the histological analysis.

Name	Species	Supplier	Reference	DF for IF
Acta1	Rabbit	Sigma	A2066	1/100
CRYM	Rabbit	Proteintech	12495-1-AP	1/200
ECM1	Rabbit	Proteintech	11521-1-ap	1/500
ETV5	Rabbit	Abcam	Ab102010	1/500
Fibronectin	Mouse	Sigma	F-6140	1/400
Gas1	Goat	R&D bio-techne	AF2644	1/100
Gli3	Goat	Santa-cruz	SC6154	1/200
Galactin1	Rabbit	GeneTex	GTX101566	1/500
Netrin	Rabbit	Abcam	ab126729	1/500
Olig1	Goat	R&D	AF2417	1/100
OSMR	Goat	R&D	AF662-SP	1/100
P-EGFR	Rabbit	Abcam	AB68470	1/250
p-Erk	Rabbit	Cell signaling	4370	1/200
P-STAT3	Rabbit	Cell signaling	TYR705	1/100
YAP	Rabbit	Cell Signaling	14074	1/100
YAP/TAZ	Rabbit	Cell Signaling	8418	1/200
TEAD	Mouse	BD	610922	1/200

Refrences

[1] A. Ribeiro, J. F. Monteiro, A. C. Certal, A. M. Cristovão, and L. Saúde, "Foxj1a is expressed in ependymal precursors, controls central canal position and is activated in new ependymal cells during regeneration in zebrafish," *Open Biol.*, 2017.

[2] B. Chermuła *et al.*, "New Gene Markers of Angiogenesis and Blood Vessels Development in Porcine Ovarian Granulosa Cells during Short-Term Primary Culture in Vitro," *Biomed Res. Int.*, 2019.

[3] M. J. Cardozo, K. S. Mysiak, T. Becker, and C. G. Becker, "Reduce, reuse, recycle – Developmental signals in spinal cord regeneration," *Developmental Biology*. 2017.

[4] C. A. Grégoire, B. L. Goldenstein, E. M. Floriddia, F. Barnabé-Heider, and K. J. L. Fernandes, "Endogenous neural stem cell responses to stroke and spinal cord injury," *GLIA*. 2015.

[5] H. Sabelström, M. Stenudd, and J. Frisén, "Neural stem cells in the adult spinal cord," *Experimental Neurology*. 2014.

[6] M. A. Anderson *et al.*, "Astrocyte scar formation AIDS central nervous system axon regeneration," *Nature*, 2016.

[7] F. Barnabé-Heider and J. Frisén, "Stem cells for spinal cord repair," *Cell Stem Cell*. 2008.

[8] Y. Qin, W. Zhang, and P. Yang, "Current states of endogenous stem cells in adult spinal cord," *J. Neurosci. Res.*, 2015.

[9] C. G. Becker, T. Becker, and J. P. Hugnot, "The spinal ependymal zone as a source of endogenous repair cells across vertebrates," *Progress in Neurobiology*. 2018.

[10] B. Paniagua-Torija *et al.*, "Cells in the adult human spinal cord ependymal region do not proliferate after injury," *J. Pathol.*, 2018.

[11] Y. Lallemand, J. Moreau, C. Saint Cloment, F. L. Vives, and B. Robert, "Generation and characterization of a tamoxifen inducible Msx1CreERT2knock-in allele," *Genesis*, 2013.

[12] H. N. Noristani, J. C. Sabourin, H. Boukhaddaoui, E. Chan-Seng, Y. N. Gerber, and F. E. Perrin, "Spinal cord injury induces astroglial conversion towards neuronal lineage," *Mol. Neurodegener.*, 2016.

[13] J. E. Lee and J. G. Gleeson, "Cilia in the nervous system: Linking cilia function and neurodevelopmental disorders," *Curr. Opin. Neurol.*, 2011.

[14] G. L. O'Grady *et al.*, "Recessive ACTA1 variant causes congenital muscular dystrophy with rigid spine," *Eur. J. Hum. Genet.*, 2015.

[15] S. Obayashi, H. Tabunoki, S. U. Kim, and J. I. Satoh, "Gene expression profiling of human neural progenitor cells following the serum-induced astrocyte differentiation," *Cell. Mol. Neurobiol.*, 2009.

[16] A. Arzan Zarin and J. P. Labrador, "Motor axon guidance in Drosophila," *Seminars in Cell and Developmental Biology*. 2019.

[17] M. Namihira and K. Nakashima, "Mechanisms of astrocytogenesis in the mammalian brain," *Current Opinion in Neurobiology*. 2013.

[18] J. J. Breunig *et al.*, "Ets Factors Regulate Neural Stem Cell Depletion and Gliogenesis in Ras Pathway Glioma," *Cell Rep.*, 2015.

[19] S. Tamura, Y. Morikawa, and E. Senba, "Up-regulated phosphorylation of signal transducer and activator of transcription 3 and cyclic AMP-responsive element binding protein by peripheral inflammation in primary afferent neurons possibly through oncostatin M receptor," *Neuroscience*, 2005.

[20] T. Taga, K. Ito, A. Noguchi, H. Arakawa, Y. Uosaki, and T. Takizawa, " Gfap and Osmr regulation by BRG1 and STAT3 via interchromosomal gene clustering in astrocytes ," *Mol. Biol. Cell*, 2017.

[21] A. Lavado *et al.*, "The Hippo Pathway Prevents YAP/TAZ-Driven Hypertranscription and Controls Neural Progenitor Number," *Dev. Cell*, 2018.
[22] V. Moreno-Manzano *et al.*, "Activated spinal cord ependymal stem cells rescue neurological function," *Stem Cells*, 2009.
[23] K. Pawar *et al.*, "Biomaterial bridges enable regeneration and re-entry of corticospinal tract axons into the caudal spinal cord after SCI: Association with recovery of forelimb function," *Biomaterials*, 2015.
[24] E. F. Schmidt, L. Kus, S. Gong, and N. Heintz, "BAC transgenic mice and the GENSAT database of engineered mouse strains," *Cold Spring Harb. Protoc.*, 2013.
[25] D. Mizrak *et al.*, "Single-Cell Analysis of Regional Differences in Adult V-SVZ Neural Stem Cell Lineages," *Cell Rep.*, 2019.
[26] C. M. O'Hara, M. W. Egar, and E. A. G. Chernoff, "Reorganization of the ependyma during axolotl spinal cord regeneration: Changes in intermediate filament and fibronectin expression," *Dev. Dyn.*, 1992.
[27] M. H. Tuszynski, R. Grill, L. L. Jones, H. M. McKay, and A. Blesch, "Spontaneous and augmented growth of axons in the primate spinal cord: Effects of local injury and nerve growth factor-secreting cell grafts," *J. Comp. Neurol.*, 2002.
[28] V. R. King, A. Alovskaya, D. Y. T. Wei, R. A. Brown, and J. V. Priestley, "The use of injectable forms of fibrin and fibronectin to support axonal ingrowth after spinal cord injury," *Biomaterials*, 2010.
[29] R. Harris, L. M. Sabatelli, and M. A. Seeger, "Guidance cues at the Drosophila CNS midline: Identification and characterization of two Drosophila Netrin/UNC-6 homologs," *Neuron*, 1996.
[30] K. Keleman and B. J. Dickson, "Short- and long-range repulsion by the Drosophila Unc5 Netrin receptor," *Neuron*, 2001.
[31] K. L. Ring *et al.*, "Genomic Analysis Reveals Disruption of Striatal Neuronal Development and Therapeutic Targets in Human Huntington's Disease Neural Stem Cells," *Stem Cell Reports*, 2015.
[32] M. T. Cobourne, "Restriction of sonic hedgehog signalling during early tooth development," *Development*, 2004.
[33] C. S. Lee, N. R. May, and C. M. Fan, "Growth arrest specific gene 1 is a positive growth regulator for the cerebellum," *Dev. Biol.*, 2001.
[34] B. L. Allen, T. Tenzen, and A. P. McMahon, "The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development," *Genes Dev.*, 2007.
[35] V. Ribes *et al.*, "Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube," *Genes Dev.*, 2010.
[36] C. B. Bai and A. L. Joyner, "Gli1 can rescue the in vivo function of Gli2.," *Development*, 2001.
[37] C. B. Bai, W. Auerbach, J. S. Lee, D. Stephen, and A. L. Joyner, "Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway.," *Development*, 2002.
[38] J. Motoyama, L. Milenkovic, M. Iwama, Y. Shikata, M. P. Scott, and C. C. Hui, "Differential requirement for Gli2 and Gli3 in ventral neural cell fate specification," *Dev. Biol.*, 2003.
[39] C. B. Bai, D. Stephen, and A. L. Joyner, "All mouse ventral spinal cord patterning by Hedgehog is Gli dependent and involves an activator function of Gli3," *Dev. Cell*, 2004.
[40] P. Mill *et al.*, "Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development," *Genes Dev.*, 2003.
[41] B. Luu, S. Ahn, G. Ge, H. Wang, and Y. Uchida, "Gli3 Is Required for Maintenance and Fate Specification of Cortical Progenitors," *J. Neurosci.*, 2011.
[42] S. Ahn and A. L. Joyner, "Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning," *Cell*, 2004.

[43] S. Ahn and A. L. Joyner, “In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog,” *Nature*, 2005.
[44] Z. Chaker, P. Codega, and F. Doetsch, “A mosaic world: puzzles revealed by adult neural stem cell heterogeneity,” *Wiley Interdisciplinary Reviews: Developmental Biology*. 2016.
[45] H. Wang, A. W. Kane, C. Lee, and S. Ahn, “Gli3 repressor controls cell fates and cell adhesion for proper establishment of neurogenic niche,” *Cell Rep.*, 2014.
[46] S. A. Vokes, H. Ji, W. H. Wong, and A. P. McMahon, “A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb,” *Genes Dev.*, 2008.
[47] K. Nakashima and T. Taga, “Mechanisms underlying cytokine-mediated cell-fate regulation in the nervous system,” *Mol. Neurobiol.*, 2002.
[48] H. Asano, M. Aonuma, T. Sanosaka, J. Kohyama, M. Namihira, and K. Nakashima, “Astrocyte differentiation of neural precursor cells is enhanced by retinoic acid through a change in epigenetic modification,” *Stem Cells*, 2009.
[49] C. Stuhlmann-Laeisz, “Forced Dimerization of gp130 Leads to Constitutive STAT3 Activation, Cytokine-independent Growth, and Blockade of Differentiation of Embryonic Stem Cells,” *Mol. Biol. Cell*, 2006.
[50] R. A. Korsak *et al.*, “STAT3 is a Critical Regulator of Astrogliosis and Scar Formation after Spinal Cord Injury,” *J. Neurosci.*, 2008.
[51] J. E. Herrmann *et al.*, “STAT3 is a Critical Regulator of Astrogliosis and Scar Formation after Spinal Cord Injury,” *J. Neurosci.*, 2008.
[52] T. Takizawa *et al.*, “DNA Methylation Is a Critical Cell-Intrinsic Determinant of Astrocyte Differentiation in the Fetal Brain,” *Dev. Cell*, 2001.
[53] M. Gaete *et al.*, “Spinal cord regeneration in Xenopus tadpoles proceeds through activation of Sox2-positive cells,” *Neural Dev.*, 2012.
[54] R. Muñoz, G. Edwards-Faret, M. Moreno, N. Zuñiga, H. Cline, and J. Larraín, “Regeneration of Xenopus laevis spinal cord requires Sox2/3 expressing cells,” *Dev. Biol.*, 2015.
[55] C. Mertens and J. E. Darnell, “SnapShot: JAK-STAT Signaling,” *Cell*, 2007.
[56] P. M. Vidal *et al.*, “Oncostatin M Reduces Lesion Size and Promotes Functional Recovery and Neurite Outgrowth After Spinal Cord Injury,” *Mol. Neurobiol.*, 2014.
[57] E. A. Goldstein, Z. C. Hesp, C. J. Miranda, B. K. Kaspar, and D. M. McTigue, “Chronic Oligodendrogenesis and Remyelination after Spinal Cord Injury in Mice and Rats,” *J. Neurosci.*, 2015.
[58] K. W. Park, C. Y. Lin, and Y. S. Lee, “Expression of Suppressor of Cytokine Signaling-3 (SOCS3) and its role in neuronal death after complete spinal cord injury,” *Exp. Neurol.*, 2014.
[59] I. B. Wanner *et al.*, “Glial Scar Borders Are Formed by Newly Proliferated, Elongated Astrocytes That Interact to Corral Inflammatory and Fibrotic Cells via STAT3-Dependent Mechanisms after Spinal Cord Injury,” *J. Neurosci.*, 2013.
[60] M. A. Anderson *et al.*, “Astrocyte scar formation aids CNS axon regeneration,” *Nature*, 2016.
[61] D. Jin, Y. Liu, F. Sun, X. Wang, X. Liu, and Z. He, “Restoration of skilled locomotion by sprouting corticospinal axons induced by co-deletion of PTEN and SOCS3,” *Nat. Commun.*, 2015.
[62] V. S. Tapia, M. Herrera-Rojas, and J. Larrain, “ JAK-STAT pathway activation in response to spinal cord injury in regenerative and non-regenerative stages of Xenopus laevis ,” *Regeneration*, 2017.
[63] C. Lang, P. M. Bradley, A. Jacobi, M. Kerschensteiner, and F. M. Bareyre, “STAT3 promotes corticospinal remodelling and functional recovery after spinal cord injury,” *EMBO Rep.*, 2013.
[64] A. A. Oliva, Y. Kang, J. Sanchez-Molano, C. Furones, and C. M. Atkins, “STAT3 signaling after

traumatic brain injury," *J. Neurochem.*, 2012.
[65] I. Glezer and S. Rivest, "Oncostatin M is a novel glucocorticoid-dependent neuroinflammatory factor that enhances oligodendrocyte precursor cell activity in demyelinated sites," *Brain, Behavior, and Immunity*. 2010.
[66] X. Xia *et al.*, "Oncostatin M protects rod and cone photoreceptors and promotes regeneration of cone outer segment in a rat model of retinal degeneration," *PLoS One*, 2011.
[67] K. Rychli *et al.*, "The inflammatory mediator oncostatin M induces angiopoietin 2 expression in endothelial cells in vitro and in vivo," *J. Thromb. Haemost.*, 2010.
[68] H. Slaets *et al.*, "Oncostatin M Reduces Lesion Size and Promotes Functional Recovery and Neurite Outgrowth After Spinal Cord Injury," *Mol. Neurobiol.*, 2014.
[69] B. Zhao, L. Li, Q. Lei, and K. L. Guan, "The Hippo-YAP pathway in organ size control and tumorigenesis: An updated version," *Genes and Development*. 2010.
[70] D. Pan, "The hippo signaling pathway in development and cancer," *Developmental Cell*. 2010.
[71] F. X. Yu and K. L. Guan, "The Hippo pathway: Regulators and regulations," *Genes and Development*. 2013.
[72] G. Halder and R. L. Johnson, "Hippo signaling: growth control and beyond," *Development*, 2010.
[73] J.-S. Mo, H. W. Park, and K.-L. Guan, "The Hippo signaling pathway in stem cell biology and cancer," *EMBO Rep.*, 2014.
[74] S. Piccolo, S. Dupont, and M. Cordenonsi, "The Biology of YAP/TAZ: Hippo Signaling and Beyond," *Physiol. Rev.*, 2014.
[75] M. A. Johnston and D. A. Lim, "Keeping Them Quiet: BMPs Maintain Adult Neural Stem Cell Quiescence," *Cell Stem Cell*, 2010.
[76] A. J. Crowther and J. Song, "Activity-dependent signaling mechanisms regulating adult hippocampal neural stem cells and their progeny," *Neuroscience Bulletin*. 2014.
[77] H. Chen *et al.*, "BMP2-SMAD Signaling Represses the Proliferation of Embryonic Neural Stem Cells through YAP," *J. Neurosci.*, 2014.
[78] Z. Huang *et al.*, "YAP stabilizes SMAD1 and promotes BMP2-induced neocortical astrocytic differentiation," *Development*, 2016.
[79] S. E. Hiemer and X. Varelas, "Stem cell regulation by the Hippo pathway," *Biochimica et Biophysica Acta - General Subjects*. 2013.
[80] Z. Huang, Y. Wang, G. Hu, J. Zhou, L. Mei, and W. C. Xiong, "YAP Is a Critical Inducer of SOCS3, Preventing Reactive Astrogliosis," *Cereb. Cortex*, 2016.
[81] N. Duval *et al.*, "Msx1 and Msx2 act as essential activators of Atoh1 expression in the murine spinal cord," *Development*, 2014.
[82] J. M. Lytle, R. Chittajallu, J. R. Wrathall, and V. Gallo, "NG2 cell response in the CNP-EGFP mouse after contusive spinal cord injury," *Glia*, 2009.
[83] A. R. Hackett and J. K. Lee, "Understanding the NG2 glial scar after spinal cord injury," *Frontiers in Neurology*. 2016.
[84] N. Masahira *et al.*, "Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells," *Dev. Biol.*, 2006.
[85] X. Li *et al.*, "MEK Is a Key Regulator of Gliogenesis in the Developing Brain," *Neuron*, 2012.
[86] S. Lacroix *et al.*, "Central canal ependymal cells proliferate extensively in response to traumatic spinal cord injury but not demyelinating lesions," *PLoS One*, 2014.
[87] D. Pleasure *et al.*, "Macroglial Plasticity and the Origins of Reactive Astroglia in Experimental Autoimmune Encephalomyelitis," *J. Neurosci.*, 2011.
[88] R. Tomer, L. Ye, B. Hsueh, and K. Deisseroth, "Advanced CLARITY for rapid and high-resolution imaging of intact tissues," *Nat. Protoc.*, 2014.

[89] M. Fumagalli *et al.*, “Phenotypic changes, signaling pathway, and functional correlates of GPR17-expressing neural precursor cells during oligodendrocyte differentiation,” *J. Biol. Chem.*, 2011.
[90] A. Battefeld, J. Klooster, and M. H. P. Kole, “Myelinating satellite oligodendrocytes are integrated in a glial syncytium constraining neuronal high-frequency activity,” *Nat. Commun.*, 2016.
[91] S. Szuchet *et al.*, “The genetic signature of perineuronal oligodendrocytes reveals their unique phenotype,” *Eur. J. Neurosci.*, 2011.
[92] C. Takasaki, M. Yamasaki, M. Uchigashima, K. Konno, Y. Yanagawa, and M. Watanabe, “Cytochemical and cytological properties of perineuronal oligodendrocytes in the mouse cortex,” *Eur. J. Neurosci.*, 2010.
[93] M. A. Petryniak, G. B. Potter, D. H. Rowitch, and J. L. R. Rubenstein, “Dlx1 and Dlx2 Control Neuronal versus Oligodendroglial Cell Fate Acquisition in the Developing Forebrain,” *Neuron*, 2007.
[94] S. J. Koo and S. L. Pfaff, “Fine-tuning motor neuron properties: Signaling from the periphery,” *Neuron*. 2002.
[95] M. Wegner, “Specification of Macroglia by Transcription Factors. Oligodendrocytes,” in *Comprehensive Developmental Neuroscience: Patterning and Cell Type Specification in the Developing CNS and PNS*, 2013.
[96] M. Kasai, K. Satoh, and T. Akiyama, “Wnt signaling regulates the sequential onset of neurogenesis and gliogenesis via induction of BMPs,” *Genes to Cells*, 2005.
[97] S. Kim and M. J. Webster, “Correlation analysis between genome-wide expression profiles and cytoarchitectural abnormalities in the prefrontal cortex of psychiatric disorders,” *Mol. Psychiatry*, 2010.
[98] S. Kim and M. J. Webster, “Integrative genome-wide association analysis of cytoarchitectural abnormalities in the prefrontal cortex of psychiatric disorders,” *Mol. Psychiatry*, 2011.
[99] H. Hibino, A. Inanobe, K. Furutani, S. Murakami, I. Findlay, and Y. Kurachi, “Inwardly Rectifying Potassium Channels: Their Structure, Function, and Physiological Roles -- Hibino et al. 90 (1): 291 -- Physiological Reviews,” *Physiol. Rev.*, 2010.
[100] G. S. Tomassy and V. Fossati, “How big is the myelinating orchestra? Cellular diversity within the oligodendrocyte lineage: facts and hypotheses,” *Front. Cell. Neurosci.*, 2014.
[101] S. Marques *et al.*, “Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system,” *Science (80-.).*, 2016.
[102] C. Marie *et al.*, “Oligodendrocyte precursor survival and differentiation requires chromatin remodeling by Chd7 and Chd8,” *Proc. Natl. Acad. Sci.*, 2018.

Perspectives

The importance of our study is that, it gives a clear and deep characterization of the genetic signature of the niche EZ in intact and injured spinal cord. Our study showed the major genes that are up-regulated in the activated niche, a major study to address the vital genes involved in the activation and proliferation of the quiescent EZ. So this study pave the way to address many questions regarding ependymal niche cells and NCSs and the regenerative mechanism.

An intriguing question one could assess is what are the genes implicated in the activation of the stem cell niche? And what is the function of these genes? In our study we showed the up-regulation of ETV5 following spinal cord injury. ETV5 is a member of the Ets transcription factor family, as a likely mediator of perinatal gliogenesis [18], [85]. It could be possible that the translocation of ETV5 into the nucleus is an indication of activated endogenous stem cell niche to encounter NSC depletion and enhance gliogenesis, processes tightly regulated by ETV5. So it would be interesting to mask the activity of ETV5 to confirm its function, in which using ETV5 floxed mice could be a possible way to KO the gene.

Similarly, we showed up regulation of STAT3 and the translocation of its active form p-STAT3 into the nucleus, at the same time we showed up-regulation of OSM and OSMR in the EZ following injury compared to the control. A pathway involved in astrocyte involvement in the glial scar formation, which in turn activate the inflammatory response and support axon regeneration (Deming, and Michael 2016). Also it could mediate and provide neuroprotective effect against cytotoxic injury and promotes repair in demyelinated regions after injury [65]. SO it is interesting to confirm the function of these genes both in-vivo and in-vitro. Again the use of specific gene-floxed mouse models would be a tool to knock-out the gene and validate its function in the EZ. One could check for increased severity of SCI responses in the absence of the gene. Also knocking out OSMR from cultured cells in-vitro through using Crispr-cas9 or siRNAs could also identify the function of OSMR in NSCs. Interestingly, the other way round could be done through overexpressing one of these genes in purpose to ameliorate defects following injury through the activation of the endogenous stem cell pool. For example, injecting OSMR directly into the spinal cord could be one possible method to check for OSMR over-expression effect on the EZ. Or the use of OSMR-AAV injection into the V-SVZ could be a better second method to over-express OSMR. In this manner we can check for OSMR effect on activating the quiescent EZ and check for cell fate after differentiation, and if OSMR could alter the dorsal-ventral regionalization within the EZ. Following up on what mentioned before about YAP, YAP/TAZ, and TEAD, in which they control proliferation in various organs including

the V-SVZ NSCs in the brain. There function is still unclear in the quiescent EZ in intact and injured spinal cord. So similar methods are followed recently in the lab, using the YAP-floxed mice and over-expression of YAP by lentivirus. Thus i twill allow to identify and unravel the function of this complex in the spinal cord EZ NSCs.

One of the in intriguing questions is to which extent endogenous stem cells are recruited after lesion or diseases? Few studies focused on the endogenous stem cell pool changes in degenerative diseases. In order to answer this question one could look and use mouse models of ALS, SMA, and MS, this allows to define differences and similarities in cellular responses and environmental cues. For example MS is a demyelinating inflammatory chronic disease that can be studied in EAE mice. There is little evidence about reactive ependymal cells to EAE, but previous studies already assessed proliferation activity [86], or fate mapping methods that covered a small subset of ependymal cells [87]. So it would be interesting to assess if ependymal cells are activated and recruited in EAE or any other degenerative model, and study whether they contribute to ameliorate the symptoms of the disease. Thus they could be an efficient therapeutic target to treat degenerative diseases.

In the context of the glial scar and the ability to modulate the scar in a beneficial way, first it's crucial to understand the detailed view of the scar environment and the distinct domains of the scar. This is related to a new findings of the existence of 5 subtypes of astrocytes in V-SVZ NSC niche in the brain [25]. One of the subtypes is astrocytes type-2 that have a high expression of CRYM, a protein highly up-regulated in the EZ niche of the injured spinal cord (paper 2). So is the glial scar mainly derived of Astro2 cells or it's a more heterogenic zone? Is CRYM up-regulation derive the proliferation of the Astro2 cells and derive their migration into the glial scar? So the use of Crym transgenic mouse model would allow to follow the EZ CRYM+ cells after injury and assess their response to spinal cord injury. Thus these cells can be targeted to modulate the injury environment as a future therapeutic approach.

Recent study published by the lab of Fiona Doestch showed that there is a functional heterogeneity in the NSC niche of the V-SVZ. This study showed that the lateral wall is more neurogenic producing neural progenies while septal wall is more gliogenic in which it generates glial progenies [25]. In the first paper, we showed a dorsal-ventral regionalization and heterogeneity in the EZ, also we showed in-vitro that dorsal cells (MSX1+ cells) generate both astrocytes and oligodendrocytes. This raises the question if individual ependymal cells are

multipotent in vivo and if the same ependymal cells generate scar-forming astrocytes and oligodendrocytes? Also is there a neurogenic/gliogenic potential of the dorsal-ventral EZ following injury? To address the first part of the question one could do in vivo clonal cell analysis of individual ependymal cells. That's why we started with evaluating the response of quiescent dorsal MSX1+ cells to injury and assess whether they can generate both astrocytes and oligodendrocytes after injury. A perspective was mention in the first part. Now to answer the second part of the study regarding dorsal-ventral potential of neurogenic/gliogenic potential it will be interesting to follow the same approach of the first publication. So laser micro-dissection of the dorsal, later, and ventral parts of the EZ after SCI coupled with the same RNA profiling approach used before will allow to identify the genetic signature of the different parts allowing to identify the neurogenic/gliogenic potential of each EZ part if it exist. This RNA profiling approach is not the best to be used in such study. What would be more interesting and precise is the use of single cell RNA sequencing of cells sorted from the three various parts. The use of various transgenic mouse models we have in the lab and the creation of double transgenic models labeling various cell types in the EZ will allow to sort differentially cells from the dorsal and ventral parts. Thus such approach will give a detailed analysis of the different populations and the specific neurogenic/gliogeneic potential of each cell population following SCI.

Part III

Oligodendrocyte lineage cellular diversity in the spinal cord

Project 3

Peri-Neuronal Satellite Cells in the Central Nervous System: Isolation and Characterization

Abstract

Glial-neuronal interactions are very important mechanisms for supporting and keeping neurons intact. One of these glial cells is the satellite oligodendrocyte or so called perineuronal satellite cells (PNCs). PNCs are tightly associated to the soma of large neurons and widely spread in the grey matter of the CNS both the cortical layers as well as the spinal cord. However the cellular properties and functional roles of these unmyelinating oligodendrocytes are not yet discovered. In this study, we detected by using nestin-GFP mouse that 21% and 24% of GFP-positive cells are associated to neurons immunostained for neuronal nuclear antigen in both cortex and spinal cord respectively. Although we identified PNCs as 2', 3'-cyclic nucleotide 3'-phosphodiesterase-positive cells, but they were negative for oligodendrocyte progenitor cells specific markers and myelinating oligodendrocyte specific markers. So PNCs are neither oligodendrocyte progenitor cells (PDGFRa negative) nor myelinating oligodendrocytes (MBP negative). We designed the nestin-GFP/Olig2-Tomato mice a new tool to isolate and characterize PNCs, as well as study their interaction with motor neurons. Our data suggest that PNCs are newly formed oligodendrocyte cells that could be integrated into the glial syncytium. Also it could be implicated in neurodegenerative diseases such as multiple sclerosis and amyotrophic lateral sclerosis due to their interaction with motor neurons.

Introduction

Central nervous system (CNS) is characterized by a wide cellular diversity, in which the cells communicante together for the CNS to function [1]. In addition to stem cells, there are the neurons that constitute the core component of the CNS that are integrated in the glial syncytium formed by glial cells [2]. Astrocytes, microglias, oligodendrocyte progenitor cells (OPCs), and myelinating oligodendrocytes are well known to support and protect neurons from shearing [3]. Focusing on the role of oligodendrocytes in the CNS, it is known that OPCs will proliferate and generate mature oligodendrocyte that are able to myelinate neuron axons [4]. Recent study identified 13 subpopulations of cells that belong to the oligodendrocyte lineage showing the phenotype of each cell population using single cell approach [5]. This study excluded the satellite oligodendrocyte cells or peri-neuronal cells (PNCs). PNCs are found in the gray matter of the CNS in a satellite position with the soma of neurons. These cells are widley distributed in the cortex and spinal cord, and attached to at least two types of neurons glutamatergic sensory neurons and cholinergic motor neurons [6]. Non-proliferating PNCs could be a type of oligodendrocyte lineage cells that are not well identified. PNCs are neither OPCs nor myelinating oligodendrocyte due to the fact that these cells do not express the specific markers that corresponds to these cell stages [6], [7]. So further studies are needed to confirm whether these cells are myelinating or non myelinating cells. But in fact, PNCs express markers that may correspond to the newly formed oligodendrocytes, a stage in which the cells are arrested by signals depending on CNS needs. The function of PNCs are not well illustrated, and it is restricted to providing metabolic support [6], [7], or regulating action potential firing and preventing neuronal burst [8]. So cytochemical and cytological characteristics of PNCs are identified, but the functional characteristics are not fully discovered yet. In this study, a new approach was followed using a different transgenic mouse that could target PNCs specifically with attempts to purify those cells in-vitro and assess their function.

In this peoject we highlited that the glial-neuronal interactions are very important mechanisms for supporting and keeping neurons intact. One of these glial cells is the satellite oligodendrocyte or so called perineuronal satellite cells (PNCs). PNCs are tightly associated to the soma of large neurons and widely spread in the grey matter of the CNS both the cortical layers as well as the spinal cord. However the cellular properties and functional roles of these unmyelinating oligodendrocytes are not yet discovered. In this study, we detected by using nestin-GFP mouse model that 21% and 24% of GFP-positive cells are associated to neurons immunostained for neuronal nuclear antigen in both cortex and spinal cord respectively. Also

we identified PNCs as 2', 3'-cyclic nucleotide 3'-phosphodiesterase-positive cells. CNPase positive cells were immunonegative to other glial cells (astrocytes and microglia) as well as OPCs. This lead to the fact that PNCs are neither oligodendrocyte progenitor cells (PDGFRa) nor myelinating oligodendrocytes (MBP). For the in vitro part, we designed a new tool to isolate and characterize PNCs, as well as study their interaction with motor neurons. Our data suggest that PNCs are newly formed oligodendrocyte cells that could be integrated into the glial syncytium. Also it could be implicated in neurodegenerative diseases such as multiple sclerosis and amyotrophic lateral sclerosis due to their interaction with motor neurons.

Methods

1. Animal care and models used

Animal experiments were performed in compliance with institutional and national guidelines for animal use. All mice were in a controlled environment (12 h light/dark cycle, 22 ±2 °C). All animals were handled under pathogen-free conditions and fed chow diet *ad libitum*. For this study we used : C57BL6 Nestin-GFP mice for histological experiments and cell cuture, Msx1-CreERT2/Rosa-Loxed Tomato transgenic described before for the EDU injection, Hes 5-GFP mouse line for histological analysis, and olig2 -CreERT2/Rosa-Loxed Tomato mice (Pascale Durbec) crossed with C57BL6 Nestin-GFP mice to generate the Nestin-GFP/Olig2 tomato mice for the in vitro and RNA sequencing. Transgenic (TG) mice were identifed by PCR, males and females aged from 3-5 months were used for all experiments.

2. EdU injection and EdU assay

Four Msx1 transgenic animals (3 months ; used in paper I) were injected intraperitoneally with with EdU for 5 days twice a day (50 mg/kg), then sacrificed to assess EDU incorporation using the EDU cell proliferation kit (Base click). Spinal cord sections were washed twice with 3% bovine serum album (BSA) in phosphate buffer saline (PBS 1X) (washing solution), then permeabilized with 0.5% triton X-100 in PBS (PBST) for 20 min at room temperature (RT). Permeabilization was followed by two washing steps with the washing solution. The reaction cocktail (deionized water, 10X reaction buffer, catalyst solution, 10 mM dye azide, and 10X buffer additive) was added for 30 min and sections were protected from light. Sections are washed three times, hoechst 33342 was used as a nuclear staining, finally the slides were mounted for analysis.

3. Mice spinal cord and brain tissue preparation

Mice were anesthetized and perfused with PBS 1X followed by 4% paraformaldehyde solution (PFA) (pH 7.0). Collected and dissected spinal cords are post fixed with 4% PFA for a maximum of 90 min, and brains for maximum of 2h. Spinal cords were cryoprotected in successive 10, 20, and 30% sucrose in PBS solutions for at least 6 h, then frozen in embedding medium (OCT). While brains are washed in PBS 1X for at least 2h. Thoracic part of the mice spinal cords were sectioned coranally (20µm) on cryostat (lecia), and sections were mounted on a glass slides for IF. While brains are sectioned on vibratome (50µm) and preserved in 24 well plates for histology analysis.

Human spinal cords were collected at the Montpellier GUI de Chaulliac Hopital from various organ-donor patients or post-mortem (the post-mortem interval was less than 48h) with strict observance of the legal and institutional ethical regulations. Spinal cord blocks were directly fixed with 4% PFA for 20 min, then cryoprotected in 30% sucrose solution and then frozen in embedding medium (OCT). Spinal cords were cut into 14 µm on a cryostat and mounted on slides for IF.

4. Immunocytochemistry and immunofluorescence

Frozen spinal cord sections were cooled down to RT for 30 minutes. Spinal cord sections and brain floating sections (50 µm thick) were permeabilized PBS 1X-0.1% triton X-100 (PBST) and blocked with 5% donkey serum in PBST solution for 1 hour. Thereafter tissues were incubated with primary antibodies over night at 4˚C (table 1), and with flurophore-conjugated secondary antibodies with species-specific Alexa 594 or Alexa 488 conjugated secondary antibodies (Molecular Probe), for 1 hour at RT. Hoechst 33342 was used as nuclear staining (blue). All the stainings were conducted in biological triplicate and more, and the staining quality was invesitgated by two investigators (HG and JPH).

5. Fluorescent microscopy, image analysis and Cell counting

Images were acquired on microscope upright 2 (Zeiss Axiolmager apotome) or LSM 700 confocal microscope in Z-stack using 40x or 63x objective lenses. Images of entire spinal cord sections were taken at 20x objective lenses. Clarification of mouse spinal cord was performed with protocol described in [9] and Imaris software was used for image treatments and 3D reconstruction. Counts were done manually on at least 3 mice, using more than 6 sections per animal, and no statistical methods were used yet.

Table 1: List of the antibodies used in this project.

Name	Species	Supplier	Reference	DF for IF
NG2	Rabbit	Chemicon	ab5320	1/500
PDGFRα	Rabbit	Cell signaling	3174	1/800
Gpr17	Rabbit	Gift from David Lecca		1/500
CNPase	Rabbit	Gift from N, Chauvet		1/500
Olig2	Rabbit	IBL		1/500
Olig1	Gaot	R&D	AF2417	1/100
Sox10	Rabbit	Abcam	ab155279	1/500
NKx2,2	Mouse	Hybridoma Bank		1/500
MBP	Mouse	Merck	MAB384	1/500
PLP	Mouse	Biorad	MCA839G	1/500
GFAP	Rabbit	Dako	Z0334	1/1000
ChAT	Gaot	Gift from Cedric Raoul		1/400
NeuN	Mouse	Chemicon	MAB377	1/500
GFP	Chicken	Abcam	ab13970	1/1000
GFP	Rabbit	Abcam	ab183734	1/800

Results

Characterization of PNCs

To characterize PNCs in the CNS, Nestin-GFP mouse was used which is a good model for in-vivo studies but it is not the best one for the in-vitro purification of PNCs.

1- The study of Nestin-GFP mouse model

To characterize the GFP cells within the Nestin-GFP mouse, several antibodies were used to target various proteins specific for several cellular types. First GFP cells were distributed in the white and gray matter as well as in the central canal, a reason why this model cannot be used for the invitro culture (figure 1A). White matter GFP cells showed a wide diversity from the protein expression profile (figure 1B). GFP cells (15-20% of total GFP+ cells) in the white matter were immunopositivity for OPCs (NG2 and PDGFRa) and mature OLs (PLP and MBP) specific markers. On the other hand the majority (80-85% of total GFP+ cells) were immunopositive for markers that are specific for the immature OLs such as (CNPase, NKx2.2, Olig2, Olig1 and Sox10). In addition GPR17 presence was restricted to a small population of cells a marker of very early differentiation stage.

Moreover, the grey matter GFP cells are subdivided into GFP non-Perineuronal cells and GFP Perineuronal cells according to the morphology and cellular localization. Protein profile revealed the characteristics of both cellular types. GFP+ non-Perineuronal cells showed nearlly a similar protein profile as the white matter GFP cells (figure 1C). In both spinal cord compartments GFP+ cells were negative for astrocytic (GFAP) marker and microglial (Iba1) marker (data not shown). Detailed comparison of the spinal cord GFP cells are summarized in (figure 1D). These data suggested a diversity in the GFP population that corresponds to the oligodendrocyte lineage cells.

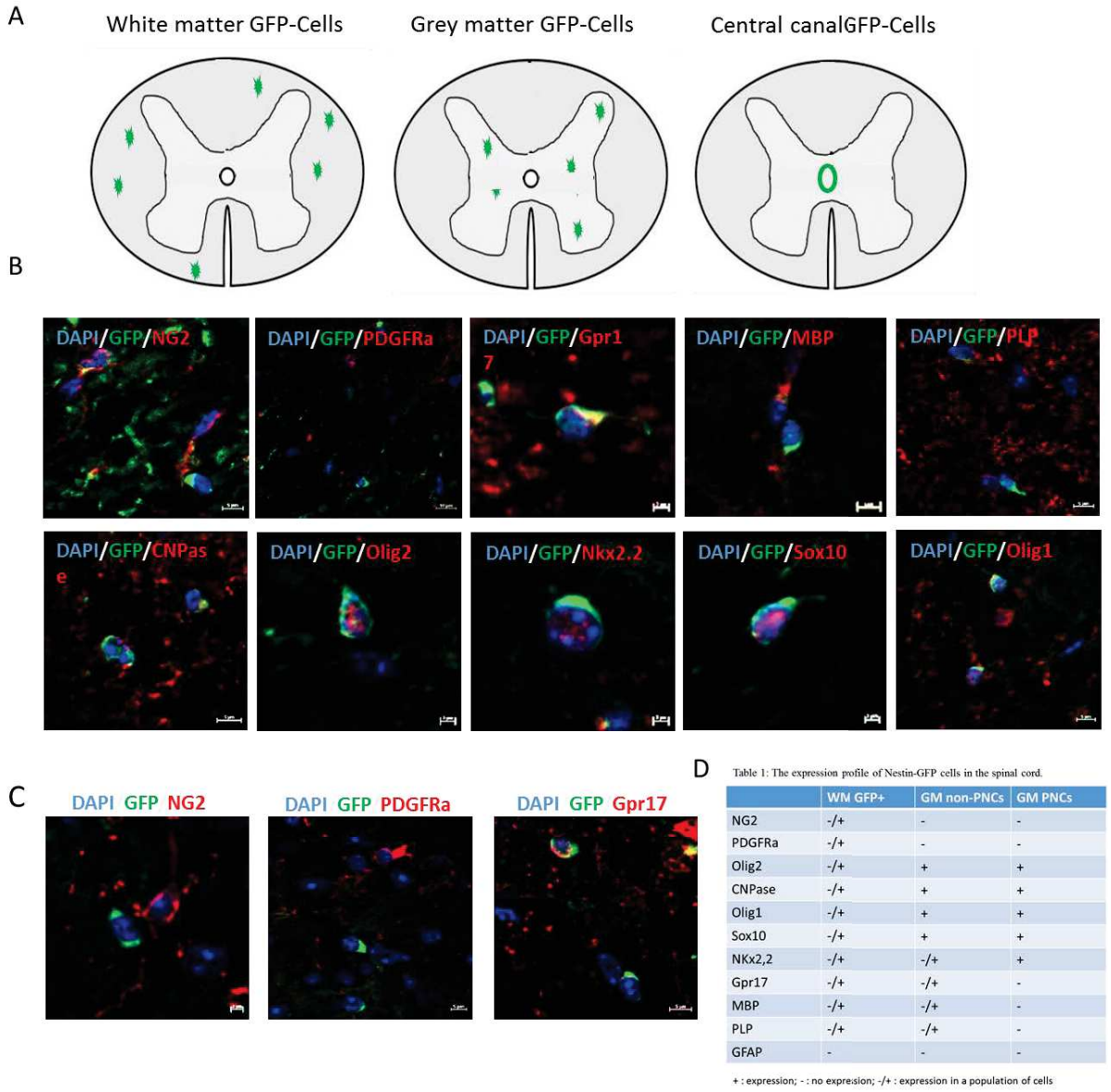


Table 1: The expression profile of Nestin-GFP cells in the spinal cord.

	WM GFP+	GM non-PNCs	GM PNCs
NG2	-/+	-	-
PDGFRa	-/+	-	-
Olig2	-/+	+	+
CNPase	-/+	+	+
Olig1	-/+	+	+
Sox10	-/+	+	+
NKx2,2	-/+	-/+	+
Gpr17	-/+	-/+	-
MBP	-/+	-/+	-
PLP	-/+	-/+	-
GFAP	-	-	-

+ : expression; - : no expression; -/+ : expression in a population of cells

Figure 1: Cellular phenotype of GFP cells in the spinal cord. (A) Schematic representation of the distribution of GFP+ cells in the white, grey matter and the central canal of the spinal cord. (B) cellular characterization of the white matter GFP+ cells. (C) cellular characterization of the white matter GFP+ cells. (D) Table summarizing the protein expression of nestin-GFP+ cells. Each marker was done three times on 3 different mice aged 3 months, with at least six tissues were analysed.

2- PNCs localized in layers IV and V of the cortex, as well as in the dorsal and ventral parts of the spinal cord

After confirming the direct association of PNCs with neurons, their distribution in the CNS was studied. As published previously, PNCs are distributed in the deep cortical layers of the cortex (layer V) [8]. But the hypothesis is that PNCs could be observed in different layers of the cortex and could be found in the spinal cord as well. So by staining neurons in the cortex and the spinal cord, PNCs exist in both compartments of the CNS. PNCs are concentrated mainly in layers IV

and V but also observed in layers III and VI of the cortex, on the other hand these cells are present in the dorsal and ventral horns of the spinal cord (figure 2).

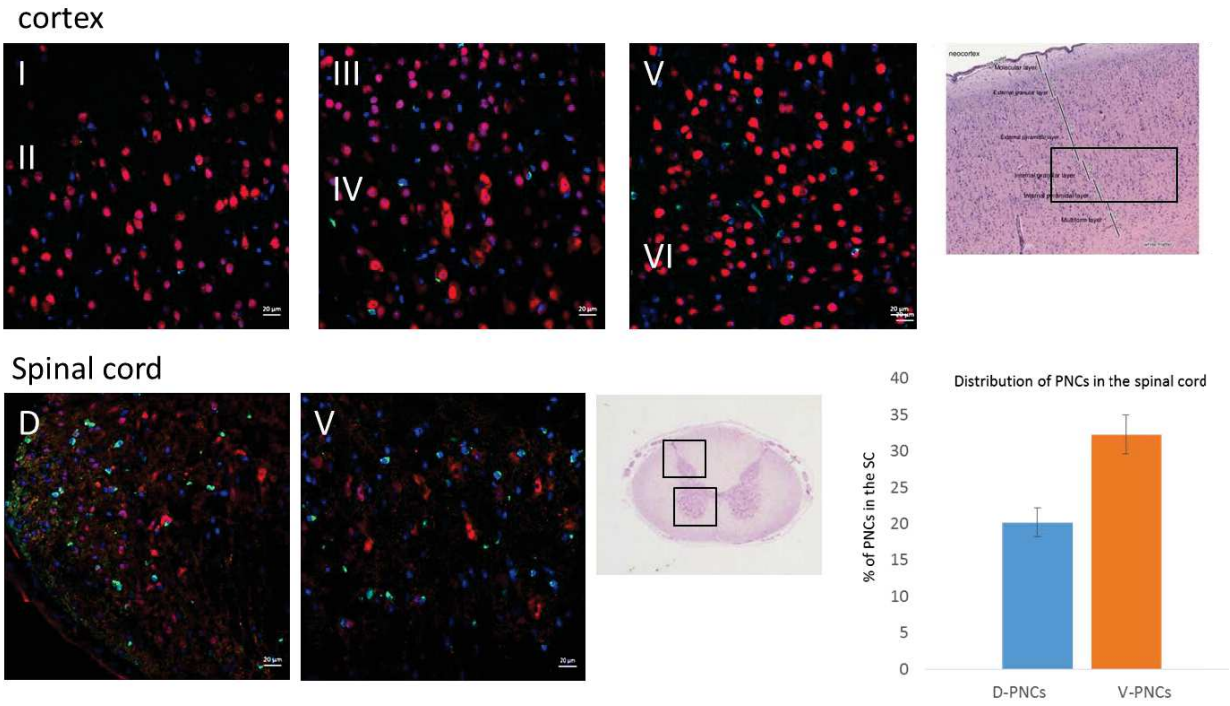


Figure 2: distribution of PNCs in the CNS. Immunostaining showing the distribution of GFP+/PNCs in the different layers of the cortex (layers III-VI), where they are much more concentrated in layers IV and V. Also GFP+/PNCs are present in the grey matter of the spinal cord in the dorsal and ventral horns, where PNCs are denser in the ventral horn. D: dorsal; V: ventral

3- PNCs interact directly with the soma of neurons in the CNS

Among the identified GFP+ cells in the grey matter we found 60% of them referred to as peri-neuronal cells (figure 3A) that were found in a close proximity with the neurons in both cortex and spinal cord grey matter. To confirm whether PNCs are in close proximity or in direct association of with neurons, neurons were stained with the neuronal marker NeuN and checked for the direct association that was obvious between the two cells (figure 3C). After quantification, 21% and 24% of the neurons are coupled to PNCs in the cortex and spinal cord respectively (figure 3B). To further confirm this direct association, brain and spinal cord were clarified and 3D reconstruction was done (data not shown). Reconstruction showed that PNCs (GFP+) were in direct contact with the soma of neurons (NeuN+) where it makes a concave shape on the neuron where it resides.

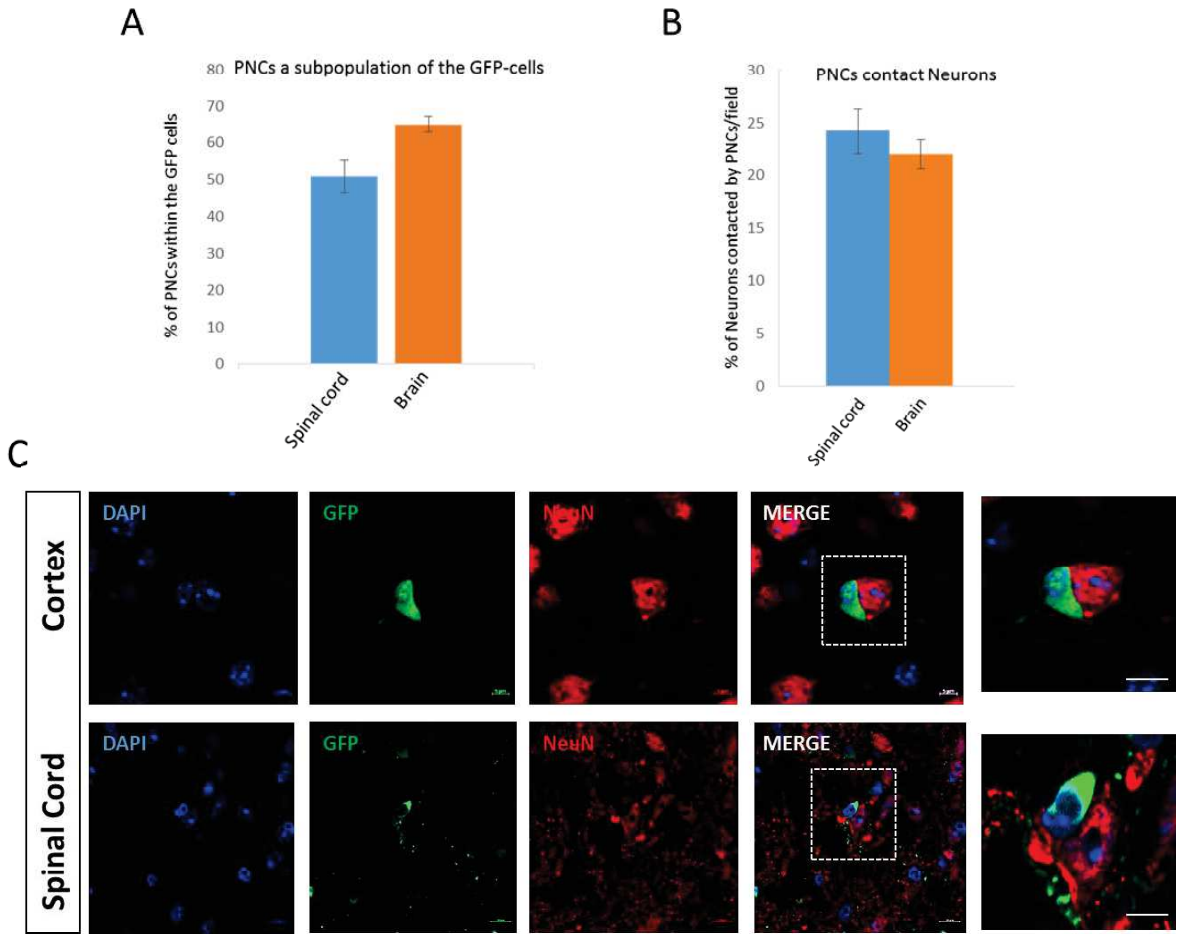


Figure 3: Attachment of PNCs with the soma of neurons in the CNS. (A) The percentage of GFP+/PNCs in the spinal cord and brain cortex. (B) Imuunofluorescence showing the GFP+ PNCs are attached to the soma of neurons stained with NeuN (red). (C) Graph showing the percentage of the neurons coupled to PNCs in spinal cord (24%) and cortex (21%).

4- PNCs are attached to different types of neurons including motor neurons

The presence of PNCs in the spinal cord was interesting and it was important to specify the types of neurons that are coupled by PNCs. As mentioned in the paper of (watanabe, M 2010) that PNCs are attached to glutamatergic neurons, which may be linked to their high expression of glutamine synthesizing enzymes. With regards to the spinal cord, their presence in the ventral horn suggest that PNCs could interact with motor neurons. To confirm this, motor neurons in the ventral horn were stained with a motor neuron specific marker, the acetylcholine transferase enzyme ChAT. Using this marker, IF confirmed that PNCs are also coupled to motor neurons, where 43% of motor neurons are associated to PNCs (figure 4). This allowed us to study in-vitro whether this interaction influence the properties and survival of motor neurons.

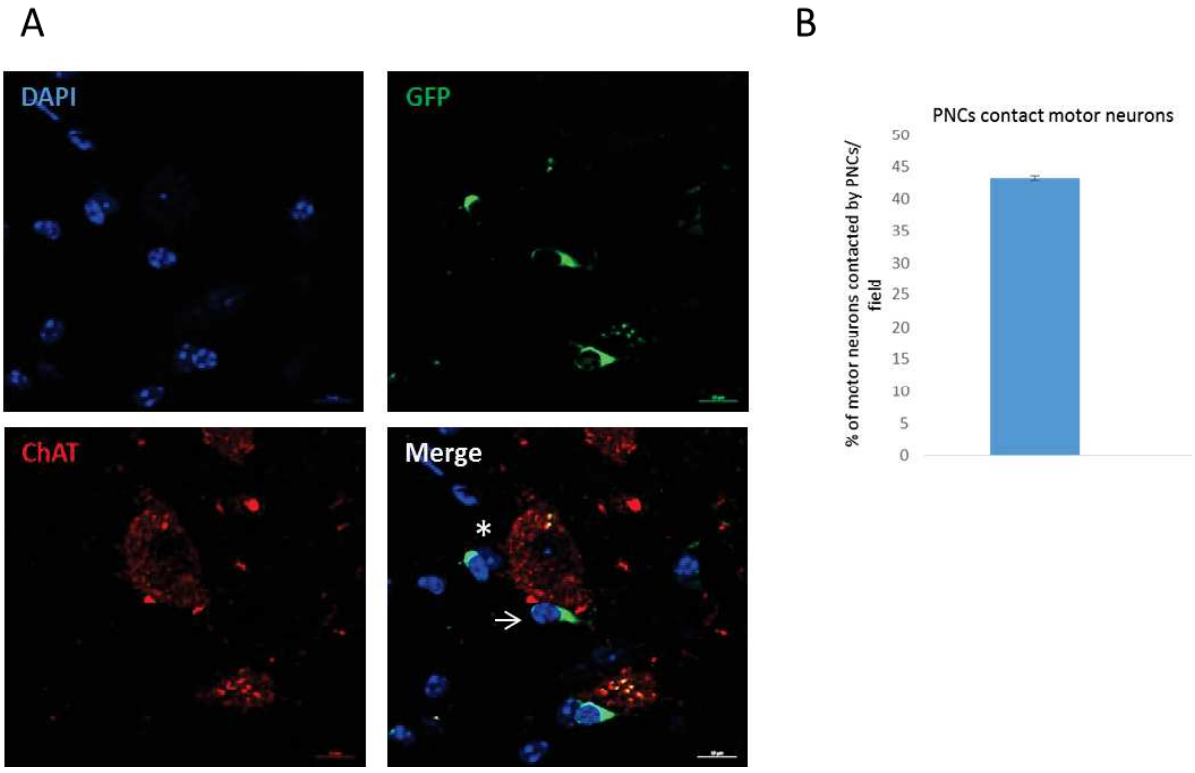
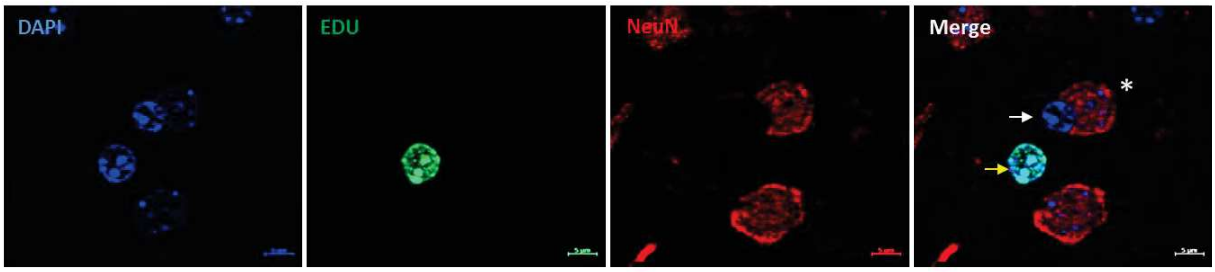


Figure 4: Attachment of PNCs to motor neurons in the spinal cord. (A) GFP+ PNCs (white arrow) are attached to the soma of motor neurons stained with NeuN (asetrix) in the ventral horn. (B) Quantification showing 43% of motor neurons are coupled to PNCs in the ventral horn of the spinal cord.

5- PNCs are non-proliferating cells

The proliferation state was assessed to check whether these cells are proliferating or resting cells. Four mice were injected intraperitoneally with EdU for one week, then brains and spinal cords were collected, sliced, and stained for EdU. PNCs where immunonegative for Edu (figure 5A), quantification on 4 different mice confirmed the immunostaining results (figure 5B). These data suggest that PNCs did not incorporate EdU unlike the progenitor cells that incorporated EdU. This make PNCs resting cells that reside in the CNS maybe to be activated by a neuronal signal that could be due to an injury or in neurodegenerative diseases.

A



B

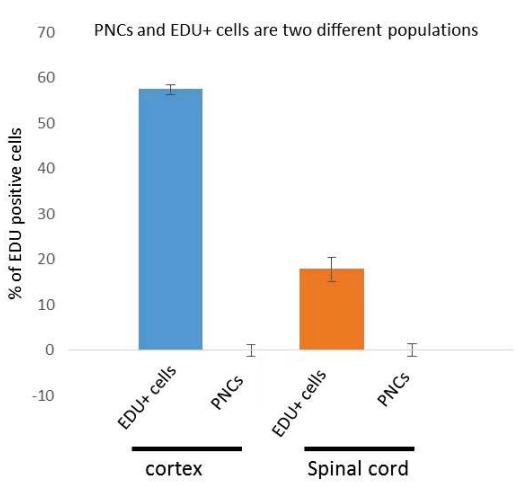


Figure 5: Proliferation state of cortical PNCs. (A) PNCS (blue DAPI-white arrow) coupled to neuron (red) didn't incorporate EDU (green- yellow arrow). (B) Graph showing the quantification of EdU incorporation of PNCs in the CNS, n= 4 mice.

6- PNCs belong to the Oligodendrocyte lineage and they could be newly formed Oligodendrocytes

PNCs were characterized by identifying the proteins expressed by these cells. Our analysis indicated that these cells are not a new type of glial cells and they are closely related to cells of the oligodendrocyte cell lineage. Their immunonegativity to neural/glial antigen 2 (NG2), and platelet derived growth factor receptor alpha (PDGFRa); myelin basic protein (MBP), and proteolipid protein (PLP) make these cells neither oligodendrocyte progenitor cells nor myelinating oligodendrocytes respectively (fig 6A, and B). On the other hand, other markers that could mark newly formed oligodendrocytes or mature non myelinating oligodendrocytes were studied. IF showed that, PNCs express CNPase, Olig1&2, NKx2.2 and Sox10 (figure 6C). PNCs were negative for other glial markers such as GFAP for astrocytes and Iba1 for microglia. This suggested that PNCs could be derived from OPCs and rested at the soma of the neurons to be activated later and give oligodendrocytes in case of oligodendrocyte depletion.

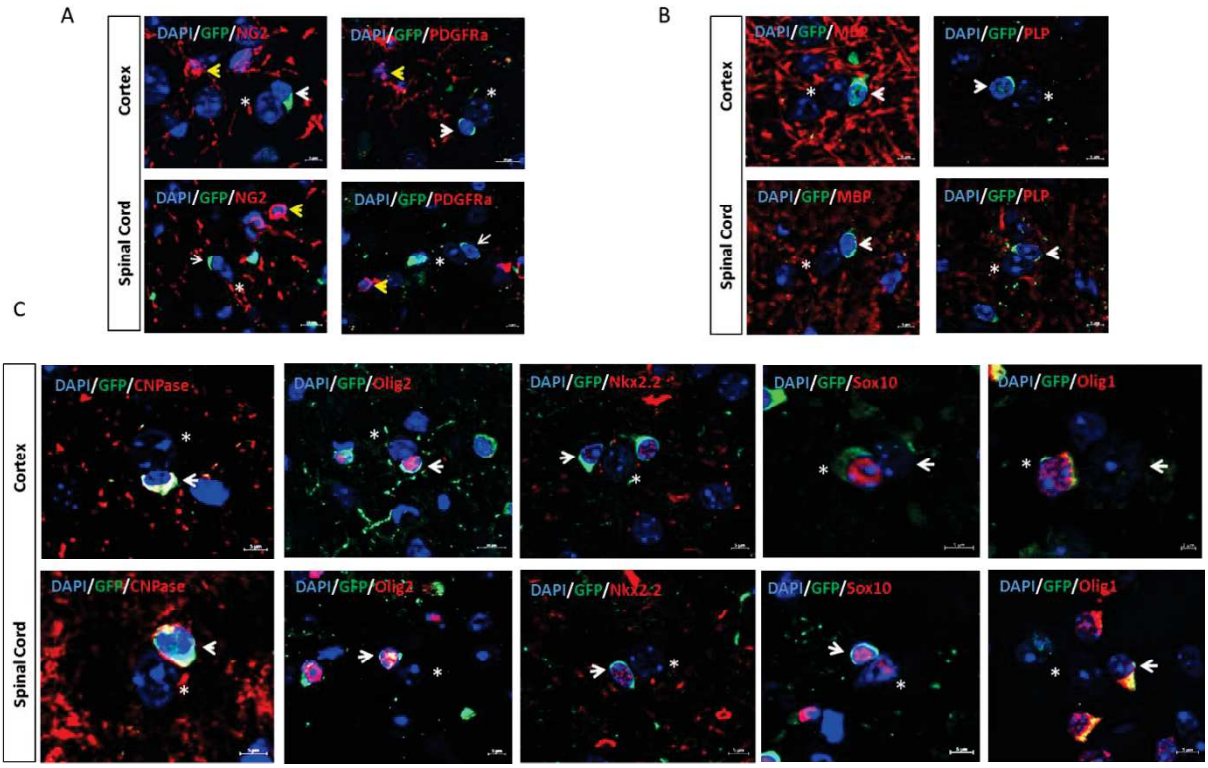


Figure 6: Characterization of PNCs in the CNS. (A) PNCs indicated in green and white in the cortex and spinal cord are immunonegative for the oligodendrocyte progenitor markers NG2 and PDGFRa. (B) PNCs indicated in green and white arrows are immunonegative for mature oligodendrocyte markers MBP and PLP in red. (C) PNCs indicated in green and white in green are immunopositive for newly formed oligodendrocyte markers such as CNPase and olig2.

7- PNCs are affected in neurodegenerative diseases

To assess whether the profile of PNCs changes in the context of degenerative diseases, we used two mouse models of SMA and ALS. We used P9, P16 mouse of SMA and wild type, these two time points refer to the pre-symptomatic and post-symptomatic stage of the disease respectively. On the other hand we used ALS and wild type mouse models aged 2 and 4 months that corresponds to the pre-symptomatic and post-symptomatic stage of the disease respectively. For each time point taken the soma of the motor neurons is still intact while degeneration is at the axonal level, so we can assess PNCs accurately. Soma of motoneurons were stained with a specific marker choline acetyltransferase (ChAT), and PNCs were labeled by Olig2 staining (Figure 7A) and CNPase (images not shown). We quantified the percentage of motoneurons coupled to PNCs, interestingly the percentage of motoneurons associated to PNCs increased in the post-symptomatic stage of SMA disease compared to the control (figure 7B). On the other hand there was an increase in the percentage of motoneurons associated to

PNCs in both pre and post-symptomatic stage of ALS disease (figure 7C). These data suggest that degenerative diseases influence the PNCs coupling to motoneurons, and this influence was at earlier stage in the ALS model.

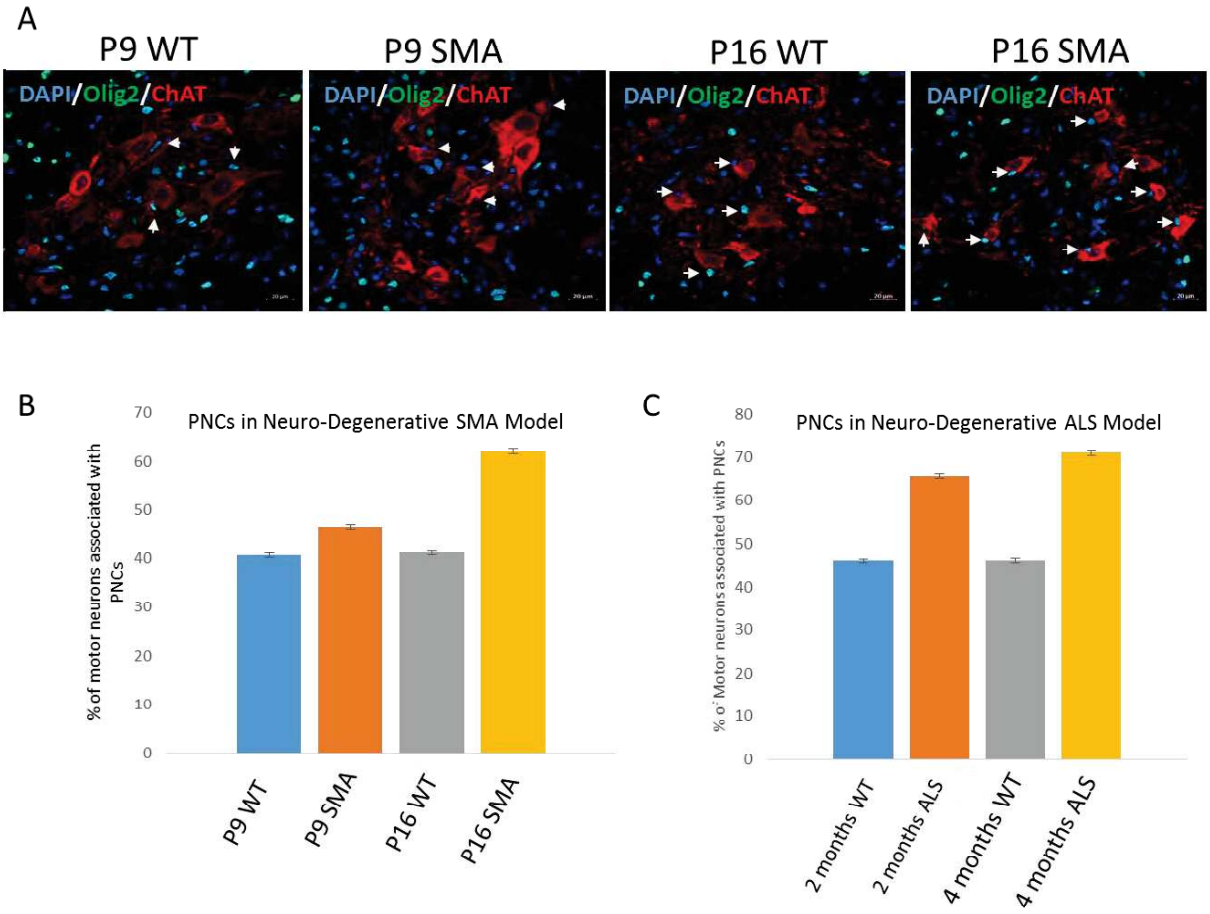


Figure 7: PNCs in the context of degenerative diseases. (A) Immunofluorescence labeling motoneurons in red and PNCs in green in SMA spinal cord sections. (B) Quantification of the % of motoneurons associated with PNCs in SMA model. (C) Quantification of the % of motoneurons associated with PNCs in ALS model. Quantifications are don on n=3.

8- Designing a new transgenic mouse model to purify PNCs

The second approach aims to ease the purification of PNCs, it was to design a new mouse model that allow the enrichment of our culture in PNCs and to deplete central canal derived GFP+ cells. So for this reason, the Nestin-GFP mouse model was crossed with a new mouse model the Olig2-tomato provided by P Durbec's lab (figure 8A). The properties of the Olig2 mouse is that the central canal cells are negative for Olig2 (figure 8B), while PNCs are positive for this marker (figure 6C). So by crossing these two mice we were expecting to highly mark PNCs and to deplete central canal cells. As expected, using this model we obtained three population of cells, Nestin-GFP, olig2-tomato, and PNCs double positive cells (figure 8A). We first

confirmed in-vivo the distribution of double positive cells, as PNCs exist in the gray matter only. So immunofluorescence showed that the majority of double positive cells reside in the grey matter and a very few minority was found in the white matter, provided by quantifying the distribution of the three population of cells in the spinal cord (figure 8 C&D). Further we confirmed that double positive cells are the PNCs and they are attached to the soma of neurons unlike the other two populations (figure 8E). Second, the main issue was to see if we will be able to sort these three populations, and indeed FACS (figure 8F) indicated that Tomato+ GFP+ were readily observed and sorted. These data validate the double transgenic Nesting/olig2 as a useful model to study PNCs in-vitro.

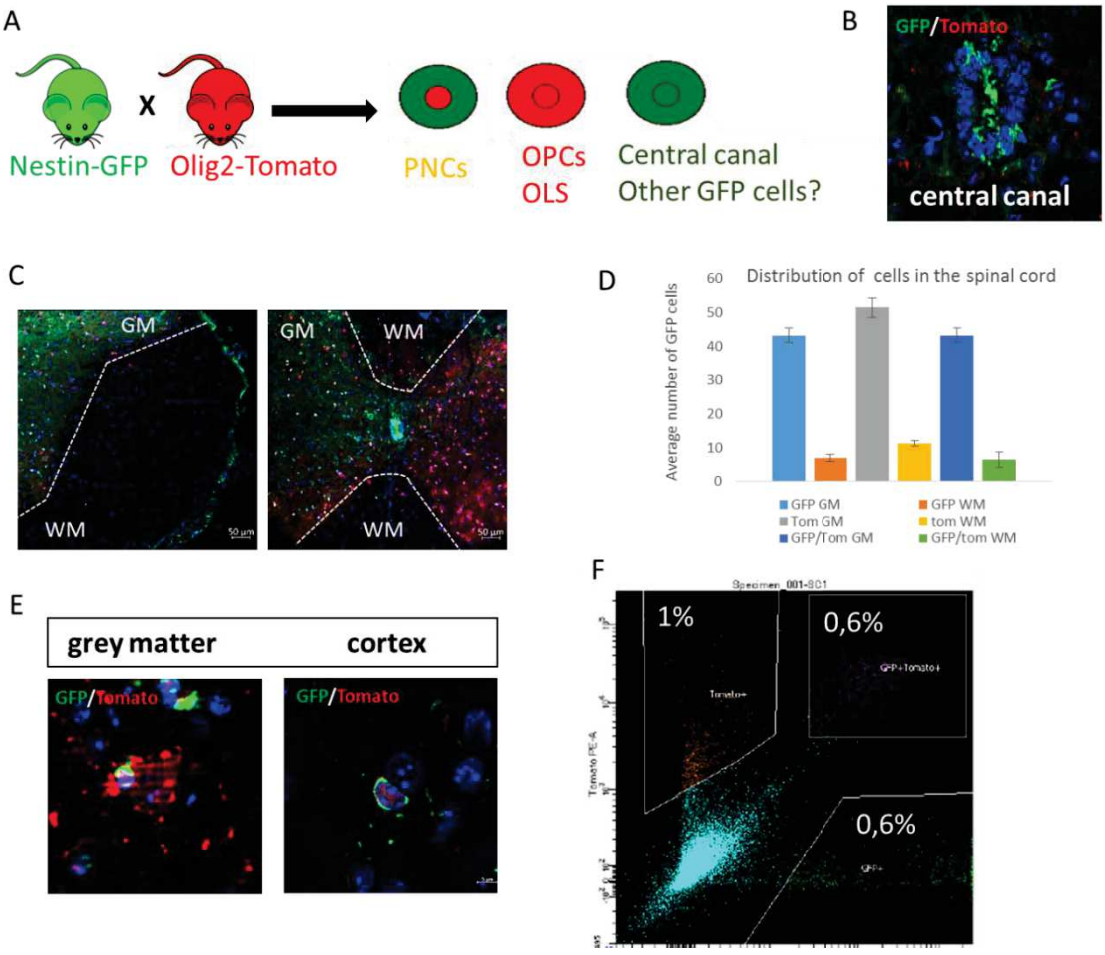


Figure 8: Creation of new mouse model for PNCs purification. (A) The schematic diagram explains the new mouse model where Nestin-GFP mouse is crossed with Olig2-Tomato mouse resulting in a mouse model that label three different cell populations. (B) GFP+ cells in the central canal are negative for Olig2/Tomato. (C) The double positive cells reside in the grey matter of the spinal cord. (D) Graph shows the distribution of the different populations in the spinal cord. (E) Double positive PNCs are attached to the soma of neurons. (F) The three different populations can be detected and sorted easily by FACS.

Discussion

In this study we aimed to characterize the protein profile of PNCs trying to unravel a specific marker to target those cells and to purify them in vitro. The outcome revealed a new poorly described cell population within the oligodendrocyte lineage. For the time being it has been confirmed that PNCs are attached to the soma of neurons in the CNS, including brain cortex and spinal cord. These cells are wide spread in different layers of the cortex and in both horns of the spinal cord. This led us to identify the different types of neurons that might be coupled with PNCs, where we identified at least two types of neurons; glutamatergic sensory neurons and cholinergic motor neurons. Protein expression profiled revealed that PNCs are neither OPCs nor OLs since they lack the specific markers of these two cell stages NG2, and PDGFRa; PLP, and MBP respectively. While these cells could be newly formed oligodendrocytes or mature non myelinating oligodendrocytes due to their expression of specific markers (CNPase, NKx2.2, Olig 1&2, and Sox10) that labels this cell stage. The presence of active Notch signaling in PNCs, as suggested by the study of Hes5-GFP mice, indicates that PNCs may be halted in their oligodendrocyte differentiation by Notch. Unraveling this code of repression could open up new approaches to elicit remyelination in demyelinating diseases such as multiple sclerosis.

Previous work has used the glutamate decarboxylase 67 (GAD67)-GFP mouse model where they referred to PNCs as CNPase+ cells [6]. In this study they used postnatal day P0-P21 a developmental stage we didn't check in our study. Similarly, they studied the distribution of PNCs in the brain cortex only focusing on the glia specific or glia enriched metabolic enzymes. Of the identified enzymes, PNCs were positive for enzymes involved in glutamate-glutamine cycle such as creatine synthetic enzyme, while they were negative for the glutamate transporters such as GLAST. This could allow PNCs to attach more to the soma of glutamatergic neurons rather than GABAergic interneurons, which were identified by specific neuronal markers. The weakness of this study is that they used a single protein the CNP to identify PNCs where CNP could be found in other OLs types not only PNCs. Therefore in-vitro purification and analysis is impossible. Also this study neglected the fact that PNCs could be attached to other neuronal cell types not only glutamatergic and GABAergic neurons, a property could allow to identify the function of these PNCs.

Beyond the cytochemical and cytological properties of PNCs [6], the genetic signature of PNCs revealed their unique phenotype. In this study they used mouse, rat, and human brains to study PNCs. CNP-GFP mouse model stained by the OTMP Ab were used to mark PNCs. A

combination of markers revealed the OLPs (A2B5+) identity of PNCs, thus they do not synthesize the myelin basic protein. The non-myelinating profile of PNCs is due to the fact that PNCs are enriched in Pea3 TF which is functionally linked to receptors and adhesion molecules. As our results and results from other labs [6] revealed the immune-negativity of PNCs to PDGFRα, microarray analysis from [7] revealed the expression of both PDGFR α & β transcripts. Both transcripts increased as OLPs transition to non-myelinating PNCs. Even though transcripts are expressed it doesn't indicate the expression of an active protein, a case that maintain cells in their specific stage. Microarray analysis from [7] uncovered a set of TFs which has been confirmed by our study such as Olig1/ 2, SOX10 TFs. The two sets of TFs are related to either acquisition of the myelinating fate (DLX1/ 2, Olig1/ 2, SOX10 and ASCL1) [11] or essential for motor neurons (LHX2, PEA3 and OTX2) [12]. The second set of cells could explain the existence of PNCs in the spinal ventral horn where they are tightly associated to motor neurons. Where the changes in the percentage of motor neurons coupled to PNCs in SMA and ALS could be explained by changes in the expression of those TFs.

Identification of Notch signaling through Hes5 expression was interesting and indicative but still how this signaling pathway is controlling PNCs fate is unknown yet. Same results were already found in [7], where they showed up regulation of Hes5 in PNCs, a Notch target and inhibitor of the myelinating genes [13]. In addition to Notch, Shh and Wnt pathways are key pathways involved in regulating PNCs fate. As described before PDGFR α is high in PNCs where this expression is regulated by Shh, also Fzd2 transcript is highly expressed by PNCs an indication of active Wnt/ β-catenin pathway. The involvement of these pathways regulate the onset of both neurogenesis and oligogenesis as well as prevent the differentiation of OLPs [14]. In our study we were unable to unravel the function of PNCs as we failed to purify these cells and we were unable to select specific marker that we can target. This was a long standing challenge to define the exact function of PNCs. In 1979 Ludwin proposed that PNCs have the ability to remyelinate denuded axons that is activated after a demylinatation machinery, which has been suggested lately by the study of [7], in which PNCs maintain untranslated copies of myelin genes as a reservoir in case of demyelination episode. Indeed, in the study of [6] they speculated that the function of PNCs as to fulfill the metabolic support to the associated cortical neurons rather than controlling synaptic transmission. In fact this refers to the idea that PNCs are attached to glutamatergic neurons and they express Glutamate enzymes, thus they are involved in the glutamate-glutamine cycle. Several reports claiming the involvement of PNCs abnormalities associated with mental disorders and they could play a role in development and homeostasis of prefrontal cortex [15], [16]. They confirmed in these reports that the number of

PNCs decreased in psychiatric disorders where it was correlated to a glutamine transporter Slc38A1, which is highly expressed in PNCs compared to myelinating OLs. This gene could be a target to understand how could PNCs be involved in the disorder diagnosis, and progression?

Functional studies continued to unravel the exact function of PNCs, using electrophysiological approaches. In this study they used PLP-ECFP-positive animals to target PNCs where in our study PNCs where negative for this protein, so this could be due to activation of the gene but not the protein at this stage. Simultaneous whole-cell patch-clamp recordings were used to investigate the function of PNCs. Results showed that PNCs are residing next to the AIS where they exhibit time-locked Ba2+ currents in response to APs from associated neurons. On the other hand the APs generated evoke inward K+ currents not through Kir4.1 channels expressed by PNCs rather by tight junction coupling PNCs and neuron. PNCs in the glial syncytium restrict APs during accumulation of high concentration of K+ in response to repetitive high frequency AP [17]. Thus PNCs-neuron coupling not only critical for axonal myelination but also for regulating neuronal excitability and protecting neurons from APs burst [8].

The emergence of single cell analysis and assessing the cellular heterogeneity within OLs lineage using this approach, led to the identification of 13 distinct populations that are diversified into six mature states within the OLs lineage [5]. Comparing the RNA-seq database to our results, it could be suggested that PNCs might belong to the newly formed OLs. These data showed a high expression of SOX10 in the NFOLs 1&2 unlike other identified cells which coincide with our results. Some of the identified genes we didn't check were Tcf7l2, Itpr2, and Tmem2. ITPR2 protein encodes for an intracellular Ca2+ channel that overlapped up to 100% with SOX10+ cells. They showed that those ITPR2+ cells differ from OPCs, but they are PDGFRa+ cells. So activation of OPCs by motor learning on a wheel for example increase the number of these cells that trigger their differentiation into ITPR2+/ SOX10+ cells. Thus this could be a strategy in which ITPR2+/ SOX10+ cells contribute to early learning by facilitating electric transmission through pre-myelinating function [5], [18]. Interestingly applying RNA-seq on the sorted cells from the Nestin/Olig2 mice we could be able to unravel the gene expression of GFP+/Tomato+ cells and compare them to the other cellular type as well as compare our database to the previous study. This could help in identifying whether PNCs could belong to the NFOLs state or they are a new sub type within the OLs lineage. Furthermore Tcf7l2, Itpr2, and Tmem2 could be used on our model to confirm their expression by PNCs where they could be specific markers of this subpopulation.

References

[1] J. Price, B. P. Williams, and M. Götz, “The Generation of Cellular Diversity in the Cerebral Cortex,” 2007.

[2] C. Giaume and X. Liu, “From a glial syncytium to a more restricted and specific glial networking,” *J. Physiol. Paris*, 2012.

[3] Y.-B. Lu *et al.*, “Viscoelastic properties of individual glial cells and neurons in the CNS,” *Proc. Natl. Acad. Sci.*, 2006.

[4] E. M. Gibson *et al.*, “Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain,” *Science (80-.).*, 2014.

[5] S. Marques *et al.*, “Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system,” *Science (80-.).*, 2016.

[6] C. Takasaki, M. Yamasaki, M. Uchigashima, K. Konno, Y. Yanagawa, and M. Watanabe, “Cytochemical and cytological properties of perineuronal oligodendrocytes in the mouse cortex,” *Eur. J. Neurosci.*, 2010.

[7] S. Szuchet *et al.*, “The genetic signature of perineuronal oligodendrocytes reveals their unique phenotype,” *Eur. J. Neurosci.*, 2011.

[8] A. Battefeld, J. Klooster, and M. H. P. Kole, “Myelinating satellite oligodendrocytes are integrated in a glial syncytium constraining neuronal high-frequency activity,” *Nat. Commun.*, 2016.

[9] R. Tomer, L. Ye, B. Hsueh, and K. Deisseroth, “Advanced CLARITY for rapid and high-resolution imaging of intact tissues,” *Nat. Protoc.*, 2014.

[10] M. Fumagalli *et al.*, “Phenotypic changes, signaling pathway, and functional correlates of GPR17-expressing neural precursor cells during oligodendrocyte differentiation,” *J. Biol. Chem.*, 2011.

[11] M. A. Petryniak, G. B. Potter, D. H. Rowitch, and J. L. R. Rubenstein, “Dlx1 and Dlx2 Control Neuronal versus Oligodendroglial Cell Fate Acquisition in the Developing Forebrain,” *Neuron*, 2007.

[12] S. J. Koo and S. L. Pfaff, “Fine-tuning motor neuron properties: Signaling from the periphery,” *Neuron*. 2002.

[13] M. Wegner, “Specification of Macroglia by Transcription Factors. Oligodendrocytes,” in *Comprehensive Developmental Neuroscience: Patterning and Cell Type Specification in the Developing CNS and PNS*, 2013.

[14] M. Kasai, K. Satoh, and T. Akiyama, “Wnt signaling regulates the sequential onset of neurogenesis and gliogenesis via induction of BMPs,” *Genes to Cells*, 2005.

[15] S. Kim and M. J. Webster, “Correlation analysis between genome-wide expression profiles and cytoarchitectural abnormalities in the prefrontal cortex of psychiatric disorders,” *Mol. Psychiatry*, 2010.

[16] S. Kim and M. J. Webster, “Integrative genome-wide association analysis of cytoarchitectural abnormalities in the prefrontal cortex of psychiatric disorders,” *Mol. Psychiatry*, 2011.

[17] H. Hibino, A. Inanobe, K. Furutani, S. Murakami, I. Findlay, and Y. Kurachi, “Inwardly Rectifying Potassium Channels: Their Structure, Function, and Physiological Roles -- Hibino et al. 90 (1): 291 -- Physiological Reviews,” *Physiol. Rev.*, 2010.

[18] C. Marie *et al.*, “Oligodendrocyte precursor survival and differentiation requires chromatin remodeling by Chd7 and Chd8,” *Proc. Natl. Acad. Sci.*, 2018.

Perspectives

With the emergence of the new mouse model (Nestin-GFP/Olig2-Tomato), it would be interesting to conduct the following experiments:

1- Purifying Nestin+/Olig2+ (PNC) cells, Olig2+ (i.e. OPC and OL) cells, GFP+ (central canal) cells, and negative (other) cells from the brain and spinal cord. This objective has been already started where we failed in exctracting good quality RNA for the RNA-seq analysis due to many complications in the sorting and RNA extraction. To avoid such complications, we suggested to follow the same purification protocol but using single cell RNA-Seq, this could be more helpful and more accurate than the RNA-seq of a bulk of sorted cells where we could miss some data. This technique will allow us to define and compare the RNA profile of PNCs and compare it with OPCs and OLs. Thus it allows to achieve a better characterization of these cells especially by identifying the specific genes that could allow to identify its function.

2- So far we have not detected any proliferation of PNCs in the normal situation. Thus, it could be possible that PNC can start proliferation in three different situations : 1) training mice on exercice wheels and check for increased PNC proliferation, through the number of GFP+/Olig2+ cells and/or incorporation of EdU. 2) a second strategy could be through crossing Nestin+/Olig2+ mice with an ALS mouse model found in the lab (SOD mice) in collaboration with Dr C Raoul to see if PNC cells react to motoneurons degeneration by proliferation. 3) to check for proliferation, migration, and differentiation in the context of spinal cord injuy

3- So far, studying PNCs in-vitro has been difficult due to the fact that there was no specific marker for these cells to be isolated and cultured. To deal with this issue we took advantage of using Nestin+/Olig2+ mice to purify PNCs to culture them in vitro and see if they are able (i) to survive and grow, (ii) or alternatively if they can dedifferenciate into OPC like cells by reexpressing PDGFRa receptor and NG2, (iii) to differentiate, interact and myelinate motoneurones.

4- As it is also important to study PNCs in the in-vivo context, it will be interesting to go through cell transplantation methods. PNCs transplantation in the spinal cord could be done in a mouse with SCI or in a shiverer mouse model (a demylination mouse model). This approach allow to address if these cells can generate mature oligodendrocytes or other cell types (notably astrocytes) upon injury or demylination condition, and to follow their fate in this context.

5- We showed previously that motoneurons support the survival of PNCs in-vitro, while PNCs has no effect on motoneurons survival. This experiment was performed on motoneurons derived from a wild type mouse. Combining the in-vivo increase of PNCs in the spinal cord of ALS and SMA models, it could be interesting to apply the same in-vitro approach but using motoneurons derived from the degenerative models and assess back the effect of PNCs on motoneuron survival.

6- PNCs could influence the growth and survival of motoneurons and the opposite might be correct and it might integrate into the glial syncytium. To study the influence of PNCs on motoneuron and vice versa, co-culture is a good approach to answer this questions. So co-culturing PNCs with and without motoneurons we could assess the effect of motoneuron on PNCs survival and growth. On the other hand co-culturing motoneurons with PNCs in the presnce and absence of neurotrophic factors we could assess whether PNCs could substitute the absence of factors and enhance motonruton survival and growth.

GENERAL CONCLUSIONS

The adult spinal cord harbors a NSC niche that is weakly studied compared to the brain NSC niches. Little is known about the EZ of the central canal that harbor this niche. In the lab we took advantage, and we aimed to characterize the EZ of adult mouse and human to unravel and answer vital questions in the field. Is their evidence of regeneration and neurogenesis in the spinal cord? In fact the adult spinal cord has limited regenerative potential, due to the quiescent state of the NSCs in the EZ. This might result in poor recovery after spinal cord injury. Therefore, this thesis sheds some light on the genetic signature of the NSC niche in the EZ where we unraveled major characteristics of the niche and identified different cell populations of different properties. Also we unraveled the genetic changes in the EZ after injury to understand the different regeneration capacity after SCI during adulthood.

In **Paper I**, RNA profiling was used to explore the adult human and mouse EZ and to build a new corpus of knowledge on this particular CNS region. Our findings revealed important and new characteristics of this poorly-defined spinal cord neural stem cell niche. We discovered that the mammalian EZ harbors a heterogenic NSC niche, in which this region is highly conserved and regionalized. The EZ regions appeared to be composed of a mosaic of cells with different embryonic origin and expressing different types of TF. This led to identify cell populations residing in the dorsal roof of the EZ as well as in the ventral floor in both mouse and human. Of the populations identified, is the dorsal roof cells that expressed MSX1 protein, those cells are present in both species and are quiescent. They are derived from the roof plate during early stages of development, and resided in the dorsal EZ during adult. We identified the protein profile of these cells and we assessed their function in-vitro. MSX1+ cells are low proliferating cells under the control of BMP6 pathway and they are multipotent they generate astrocytes and oligodendrocytes. This new corpus of knowledge on the organization and genes expressed in EZ will help to explore this adult stem cell niche further and will also be useful to shed light on ependymoma, a rare type of tumors that can arise in the human spinal cord EZ.

In **Paper II,** After addressing the genetic signature of the ependymal zone both in mouse and human and unravelling the cellular composition of this zone, for the moment the genetic signature of the EZ after injury is still lacking. And since one clinical interest is to address the effect of SCI on the endogenous stem cell activation. in accordance to the first article, RNA profiling was used to explore the adult mouse EZ in control and injured spinal cord. We built a new corpus of knowledge on this particular CNS region following an injury. Our preliminary data revealed important and new characteristics of the activated stem cell niche following an injury compared to the quiescent niche. Our data revealed an up-regulation of genes implicated

in increased proliferation, adhesion, axon guidance and astrogenesis. Thus the EZ stem cell niche is an active zone and a territory of endogenous stem cells that are implicated in the repair mechanism.

In Part III, I focused on the progenitor cells that could be derived from the EZ but resided in the parenchyma of the spinal cord. In this part of the study I focused on the satellite peri-neuronal cells that are neglected for a long time ago. In this study we sought to characterize the protein profile of PNCs trying to unravel a specific marker to target those cells. The outcome revealed a new poorly described cell population within the oligodendrocyte lineage. For the time being we confirmed that PNCs are attached to the soma of neurons in the CNS, including brain cortex and spinal cord. These cells are wide spread in different layers of the cortex and in both horns of the spinal cord. This led us to know the different types of neurons that might be coupled with PNCs where we identified at least two types of neurons; glutamatergic sensory neurons and cholinergic motor neurons. Protein expression profiled revealed that PNCs are neither OPCs nor OLs since they lack the specific markers of these two cell stages NG2, and PDGFRa; PLP, and MBP respectively. While these cells could be newly formed oligodendrocytes or mature non myelinating oligodendrocytes due to their expression of specific markers (CNPase, NKx2.2, Olig 1&2, and Sox10) that labels this cell stage. The presence of active Notch signaling in PNCs, as suggested by the study of pHes5-GFP mice, indicates that PNCs may be halted in their oligodendrocyte differentiation by Notch. Unraveling this code of repression could open up new approaches to elicit remyelination in demyelinating diseases such as multiple sclerosis or SMA and ALS in which I found an increase in the number of motorneurons coupled to PNCs.

Finally we can conclude that the human and mouse Spinal cord is a territory of diverse neural stem/progenitor cells, with different properties, phenotypes and functionality, In which they can be targeted as a therapeutic approach for injury repair and functional recovery in degenerative diseases.

REFERENCES

[1] N. Chuang *et al.*, “An MRI-based atlas and database of the developing mouse brain,” *Neuroimage*, 2011.
[2] H. Huang *et al.*, “Anatomical Characterization of Human Fetal Brain Development with Diffusion Tensor Magnetic Resonance Imaging,” *J. Neurosci.*, 2009.
[3] J. Nolte and J. B. Angevine Jr, “The Human Brain in Photographs and Diagrams,” *Hum. Brain Photogr. Diagrams*, 2013.
[4] W. A. Alaynick, T. M. Jessell, and S. L. Pfaff, “SnapShot: Spinal cord development,” *Cell*, 2011.
[5] S. J. Harkema, “Plasticity of interneuronal networks of the functionally isolated human spinal cord,” *Brain Research Reviews*. 2008.
[6] J. Cohen-Adad, “High-Resolution DWI in Brain and Spinal Cord with syngo RESOLVE,” *Siemens Magnetom - Clin. Neurol.*, 2012.
[7] S. Duty and P. Jenner, “Animal models of Parkinson’s disease: A source of novel treatments and clues to the cause of the disease,” *British Journal of Pharmacology*. 2011.
[8] B. H. Dobkin and L. A. Havton, “Basic Advances and New Avenues in Therapy of Spinal Cord Injury,” *Annu. Rev. Med.*, 2004.
[9] M. T. Armentero, A. Pinna, S. Ferré, J. L. Lanciego, C. E. Müller, and R. Franco, “Past, present and future of A2A adenosine receptor antagonists in the therapy of Parkinson’s disease,” *Pharmacol. Ther.*, 2011.
[10] J. Kjell and L. Olson, “Rat models of spinal cord injury: from pathology to potential therapies,” *Dis. Model. Mech.*, 2016.
[11] B. Mota and S. Herculano-Houzel, “All brains are made of this: a fundamental building block of brain matter with matching neuronal and glial masses,” *Front. Neuroanat.*, 2014.
[12] B. A. Barres, “The mystery and magic of glia: a perspective on their roles in health and disease.,” *Neuron*, 2008.
[13] V. V. Gusel’nikova and D. E. Korzhevskiy, “NeuN as a neuronal nuclear antigen and neuron differentiation marker,” *Acta Naturae*. 2015.
[14] L. Dehmelt, P. Nalbant, W. Steffen, and S. Halpain, “A microtubule-based, dynein-dependent force induces local cell protrusions: Implications for neurite initiation,” *Brain Cell Biol.*, 2006.
[15] M. Tavazoie *et al.*, “A Specialized Vascular Niche for Adult Neural Stem Cells,” *Cell Stem Cell*, 2008.
[16] Y. Ota, A. T. Zanetti, and R. M. Hallock, “The role of astrocytes in the regulation of synaptic plasticity and memory formation,” *Neural Plasticity*. 2013.
[17] J.-P. Michalski and R. Kothary, “Oligodendrocytes in a Nutshell,” *Front. Cell. Neurosci.*, 2015.
[18] F. Pérez-Cerdá, M. V. Sánchez-Gómez, and C. Matute, “Pío del Río Hortega and the discovery of the oligodendrocytes,” *Front. Neuroanat.*, 2015.
[19] A. Zeisel *et al.*, “Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq,” *Science (80-.).*, 2015.
[20] S. Marques *et al.*, “Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system,” *Science (80-.).*, 2016.
[21] A. Nishiyama, “Polydendrocytes: NG2 cells with many roles in development and repair of the CNS,” *Neuroscientist*. 2007.
[22] A. Nishiyama, R. Suzuki, and X. Zhu, “NG2 cells (polydendrocytes) in brain physiology and repair,” *Frontiers in Neuroscience*. 2014.
[23] S. C. Lin and D. E. Bergles, “Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus,” *Nat. Neurosci.*, 2004.
[24] S. H. Kang, M. Fukaya, J. K. Yang, J. D. Rothstein, and D. E. Bergles, “NG2+ CNS glial progenitors remain committed to the oligodendrocyte lineage in postnatal life and following neurodegeneration,” *Neuron*, 2010.

[25] N. Baumann and D. Pham-Dinh, “Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System,” *Physiol. Rev.*, 2001.

[26] E. Traiffort, M. Zakaria, Y. Laouarem, and J. Ferent, “Hedgehog: A Key Signaling in the Development of the Oligodendrocyte Lineage,” *J. Dev. Biol.*, 2016.

[27] R. H. Brownson, “The effect of X-irradiation on the perineuronal satellite cells in the cortex of aging brains,” *J. Neuropathol. Exp. Neurol.*, 1960.

[28] T. Abe, K. Ogawa, H. Fuziwara, K. Urayama, and K. Nagashima, “Spinal ganglia and peripheral nerves from a patient with Tay-Sachs disease - Morphological and ganglioside studies,” *Acta Neuropathol.*, 1985.

[29] C. Takasaki, M. Yamasaki, M. Uchigashima, K. Konno, Y. Yanagawa, and M. Watanabe, “Cytochemical and cytological properties of perineuronal oligodendrocytes in the mouse cortex,” *Eur. J. Neurosci.*, 2010.

[30] S. Szuchet, D. C. Plachetzki, and K. S. Eaton, “Oligodendrocyte transmembrane protein: A novel member of the glutamate-binding protein subfamily,” *Biochem. Biophys. Res. Commun.*, 2001.

[31] M. A. Petryniak, G. B. Potter, D. H. Rowitch, and J. L. R. Rubenstein, “Dlx1 and Dlx2 Control Neuronal versus Oligodendroglial Cell Fate Acquisition in the Developing Forebrain,” *Neuron*, 2007.

[32] S. Szuchet *et al.*, “The genetic signature of perineuronal oligodendrocytes reveals their unique phenotype,” *Eur. J. Neurosci.*, 2011.

[33] J. Livet *et al.*, “ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools,” *Neuron*. 2002.

[34] C. Zhao, S. P. J. Fancy, R. J. M. Franklin, and C. Ffrench-Constant, “Up-regulation of oligodendrocyte precursor cell αV integrin and its extracellular ligands during central nervous system remyelination,” *J. Neurosci. Res.*, 2009.

[35] M. Kasai, K. Satoh, and T. Akiyama, “Wnt signaling regulates the sequential onset of neurogenesis and gliogenesis via induction of BMPs,” *Genes to Cells*, 2005.

[36] T. Shimizu, T. Kagawa, T. Wada, Y. Muroyama, S. Takada, and K. Ikenaka, “Wnt signaling controls the timing of oligodendrocyte development in the spinal cord,” *Dev. Biol.*, 2005.

[37] K. Yun, S. Fischman, J. Johnson, M. Hrabe de Angelis, G. Weinmaster, and J. L. R. Rubenstein, “Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon.,” *Development*, 2002.

[38] A. Liu *et al.*, “A molecular insight of Hes5-dependent inhibition of myelin gene expression: Old partners and new players,” *EMBO J.*, 2006.

[39] K. Fujimori, K. Kadoyama, and Y. Urade, “Protein kinase C activates human lipocalin-type prostaglandin D synthase gene expression through de-repression of notch-HES signaling and enhancement of AP-2β function in brain-derived TE671 cells,” *J. Biol. Chem.*, 2005.

[40] F. Wang *et al.*, “Astrocytes modulate neural network activity by Ca2+-dependent uptake of extracellular K+,” *Sci. Signal.*, 2012.

[41] J.-P. Vit, P. T. Ohara, A. Bhargava, K. Kelley, and L. Jasmin, “Silencing the Kir4.1 Potassium Channel Subunit in Satellite Glial Cells of the Rat Trigeminal Ganglion Results in Pain-Like Behavior in the Absence of Nerve Injury,” *J. Neurosci.*, 2008.

[42] K. Baalman *et al.*, “Axon Initial Segment-Associated Microglia,” *J. Neurosci.*, 2015.

[43] M. Taniike, I. Mohri, N. Eguchi, C. T. Beuckmann, K. Suzuki, and Y. Urade, “Perineuronal Oligodendrocytes Protect against Neuronal Apoptosis through the Production of Lipocalin-Type Prostaglandin D Synthase in a Genetic Demyelinating Model,” *J. Neurosci.*, 2002.

[44] S. K. Ludwin, “The perineuronal satellite oligodendrocyte. A role in remyelination,” *Acta Neuropathol.(Berl)*, 1979.

[45] M. H. P. Kole and G. J. Stuart, “Signal Processing in the Axon Initial Segment,” *Neuron*. 2012.
[46] A. Battefeld, J. Klooster, and M. H. P. Kole, “Myelinating satellite oligodendrocytes are integrated in a glial syncytium constraining neuronal high-frequency activity,” *Nat. Commun.*, 2016.
[47] L. J. Lawson, V. H. Perry, and S. Gordon, “Turnover of resident microglia in the normal adult mouse brain,” *Neuroscience*, 1992.
[48] C. D. Aizenman and K. G. Pratt, “There’s More Than One Way to Scale a Synapse,” *Neuron*. 2008.
[49] B. Stevens *et al.*, “The Classical Complement Cascade Mediates CNS Synapse Elimination,” *Cell*, 2007.
[50] K. Meletis *et al.*, “Spinal cord injury reveals multilineage differentiation of ependymal cells,” *PLoS Biol.*, 2008.
[51] L. Xiao, C. Saiki, and R. Ide, “Stem cell therapy for central nerve system injuries: Glial cells hold the key,” *Neural Regeneration Research*. 2014.
[52] J. Slack, “Stem cells: A Very Short Introduction,” in *Stem cells: A Very Short Introduction*, 2012.
[53] T. Watabe and K. Miyazono, “Roles of TGF-β family signaling in stem cell renewal and differentiation,” *Cell Research*. 2009.
[54] M. Skardelly *et al.*, “Long-Term Benefit of Human Fetal Neuronal Progenitor Cell Transplantation in a Clinically Adapted Model after Traumatic Brain Injury,” *J. Neurotrauma*, 2011.
[55] T. Zigova *et al.*, “Neuronal progenitor cells of the neonatal subventricular zone differentiate and disperse following transplantation into the adult rat striatum,” in *Cell Transplantation*, 1998.
[56] A. J. Mothe, T. Zahir, C. Santaguida, D. Cook, and C. H. Tator, “Neural stem/progenitor cells from the adult human spinal cord are multipotent and self-renewing and differentiate after transplantation,” *PLoS One*, 2011.
[57] P. J. Horner *et al.*, “Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord.,” *J. Neurosci.*, 2000.
[58] F. Rossi and E. Cattaneo, “Neural stem cell therapy for neurological diseases: Dreams and reality,” *Nat. Rev. Neurosci.*, 2002.
[59] K. Satake, J. Lou, and L. G. Lenke, “Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue,” *Spine (Phila. Pa. 1976).*, 2004.
[60] M. Sasaki *et al.*, “BDNF-Hypersecreting Human Mesenchymal Stem Cells Promote Functional Recovery, Axonal Sprouting, and Protection of Corticospinal Neurons after Spinal Cord Injury,” *J. Neurosci.*, 2009.
[61] A. Uccelli, L. Moretta, and V. Pistoia, “Mesenchymal stem cells in health and disease,” *Nature Reviews Immunology*. 2008.
[62] S. U. Kim, H. J. Lee, and Y. B. Kim, “Neural stem cell-based treatment for neurodegenerative diseases,” *Neuropathology*. 2013.
[63] Z. Feng and F. Gao, “Stem Cell Challenges in the Treatment of Neurodegenerative Disease,” *CNS Neuroscience and Therapeutics*. 2012.
[64] M. Stenudd, H. Sabelström, and J. Frisén, “Role of endogenous neural stem cells in spinal cord injury and repair,” *JAMA Neurol.*, 2015.
[65] H. Sabelström, M. Stenudd, and J. Frisén, “Neural stem cells in the adult spinal cord,” *Experimental Neurology*. 2014.
[66] H. Ghazale *et al.*, “Docosahexaenoic acid (DHA) enhances the therapeutic potential of neonatal neural stem cell transplantation post—Traumatic brain injury,” *Behav. Brain Res.*, 2018.
[67] P. Lu, K. Kadoya, and M. H. Tuszynski, “Axonal growth and connectivity from neural stem cell grafts in models of spinal cord injury,” *Current Opinion in Neurobiology*. 2014.

[68] C. Blanpain and E. Fuchs, “Plasticity of epithelial stem cells in tissue regeneration,” *Science*. 2014.
[69] L. G. van der Flier and H. Clevers, “Stem Cells, Self-Renewal, and Differentiation in the Intestinal Epithelium,” *Annu. Rev. Physiol.*, 2009.
[70] N. Barker *et al.*, “Identification of stem cells in small intestine and colon by marker gene Lgr5,” *Nature*, 2007.
[71] N. Barker, A. Van Oudenaarden, and H. Clevers, “Identifying the stem cell of the intestinal crypt: Strategies and pitfalls,” *Cell Stem Cell*. 2012.
[72] L. Meran, A. Baulies, and V. S. W. Li, “Intestinal Stem Cell Niche: The Extracellular Matrix and Cellular Components,” *Stem Cells International*. 2017.
[73] C. A. Chacón-Martínez, J. Koester, and S. A. Wickström, “Signaling in the stem cell niche: regulating cell fate, function and plasticity,” *Development*, 2018.
[74] C. A. Chacón-Martínez, M. Klose, C. Niemann, I. Glauche, and S. A. Wickström, “Hair follicle stem cell cultures reveal self-organizing plasticity of stem cells and their progeny,” *EMBO J.*, 2017.
[75] T. Sato and H. Clevers, “SnapShot: Growing Organoids from Stem Cells,” *Cell*. 2015.
[76] K. S. Yan *et al.*, “Non-equivalence of Wnt and R-spondin ligands during Lgr5 + intestinal stem-cell self-renewal,” *Nature*, 2017.
[77] Y. C. Hsu, L. Li, and E. Fuchs, “Transit-amplifying cells orchestrate stem cell activity and tissue regeneration,” *Cell*, 2014.
[78] A. Pardo-Saganta *et al.*, “Parent stem cells can serve as niches for their daughter cells,” *Nature*, 2015.
[79] X. Lim *et al.*, “Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling,” *Science (80-.).*, 2013.
[80] J. H. Van Es *et al.*, “Dll1 + secretory progenitor cells revert to stem cells upon crypt damage,” *Nat. Cell Biol.*, 2012.
[81] H. Tian *et al.*, “Opposing activities of notch and wnt signaling regulate intestinal stem cells and gut homeostasis,” *Cell Rep.*, 2015.
[82] F. Doetsch, “A niche for adult neural stem cells,” *Current Opinion in Genetics and Development*. 2003.
[83] Y. J. Shin, J. S. Choi, J. Y. Choi, J. H. Cha, M. H. Chun, and M. Y. Lee, “Enhanced expression of vascular endothelial growth factor receptor-3 in the subventricular zone of stroke-lesioned rats,” *Neurosci. Lett.*, 2010.
[84] C. F. Calvo *et al.*, “Vascular endothelial growth factor receptor 3 directly regulates murine neurogenesis,” *Genes Dev.*, 2011.
[85] M. C. Ward and A. M. Cunningham, “Developmental expression of vascular endothelial growth factor receptor 3 and vascular endothelial growth factor C in forebrain,” *Neuroscience*, 2015.
[86] F. Lazarini and P. M. Lledo, “Is adult neurogenesis essential for olfaction?,” *Trends in Neurosciences*. 2011.
[87] B. Menn, J. M. Garcia-Verdugo, C. Yaschine, O. Gonzalez-Perez, D. Rowitch, and A. Alvarez-Buylla, “Origin of Oligodendrocytes in the Subventricular Zone of the Adult Brain,” *J. Neurosci.*, 2006.
[88] I. Kazanis, “The subependymal zone neurogenic niche: a beating heart in the centre of the brain: how plastic is adult neurogenesis? Opportunities for therapy and questions to be addressed,” *Brain*, 2009.
[89] A. van Schepdael, J. M. A. Ashbourn, R. Beard, J. J. Miller, and L. Geris, “Mechanisms of cell migration in the adult brain: modelling subventricular neurogenesis,” *Comput. Methods Biomech. Biomed. Engin.*, 2013.

[90] H. T. Ghashghaei, C. Lai, and E. S. Anton, “Neuronal migration in the adult brain: Are we there yet?,” *Nature Reviews Neuroscience*. 2007.
[91] P. A. Riquelme, E. Drapeau, and F. Doetsch, “Brain micro-ecologies: Neural stem cell niches in the adult mammalian brain,” *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2008.
[92] T. D. Palmer, A. R. Willhoite, and F. H. Gage, “Vascular niche for adult hippocampal neurogenesis,” *J. Comp. Neurol.*, 2000.
[93] T. Seki, T. Namba, H. Mochizuki, and M. Onodera, “Clustering, migration, and neurite formation of neural precursor cells in the adult rat hippocampus,” *J. Comp. Neurol.*, 2007.
[94] H. Suh, A. Consiglio, J. Ray, T. Sawai, K. A. D’Amour, and F. H. Gage, “In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of Sox2+ Neural Stem Cells in the Adult Hippocampus,” *Cell Stem Cell*, 2007.
[95] M. K. Hajihosseini *et al.*, “Localization and fate of Fgf10-expressing cells in the adult mouse brain implicate Fgf10 in control of neurogenesis,” *Mol. Cell. Neurosci.*, 2008.
[96] S. M. Sternson, “Hypothalamic survival circuits: Blueprints for purposive behaviors,” *Neuron*. 2013.
[97] A. Paul, Z. Chaker, and F. Doetsch, “Hypothalamic regulation of regionally distinct adult neural stem cells and neurogenesis,” *Science (80-.).*, 2017.
[98] R. Maggi, J. Zasso, and L. Conti, “Neurodevelopmental origin and adult neurogenesis of the neuroendocrine hypothalamus,” *Front. Cell. Neurosci.*, 2015.
[99] C. B. Johansson, S. Momma, D. L. Clarke, M. Risling, U. Lendahl, and J. Frisén, “Identification of a neural stem cell in the adult mammalian central nervous system,” *Cell*, 1999.
[100] F.-J. Obermair, A. Schroter, and M. Thallmair, “Endogenous Neural Progenitor Cells as Therapeutic Target After Spinal Cord Injury,” *Physiology*, 2008.
[101] F. Barnabé-Heider *et al.*, “Origin of new glial cells in intact and injured adult spinal cord,” *Cell Stem Cell*, 2010.
[102] L. S. Shihabuddin *et al.*, “Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus.,” *J. Neurosci.*, 2000.
[103] C. A. Grégoire, B. L. Goldenstein, E. M. Floriddia, F. Barnabé-Heider, and K. J. L. Fernandes, “Endogenous neural stem cell responses to stroke and spinal cord injury,” *GLIA*. 2015.
[104] K. Yu, S. McGlynn, and M. P. Matise, “Floor plate-derived sonic hedgehog regulates glial and ependymal cell fates in the developing spinal cord,” *Development*, 2013.
[105] C. Alfaro-Cervello, M. Soriano-Navarro, Z. Mirzadeh, A. Alvarez-Buylla, and J. M. Garcia-Verdugo, “Biciliated ependymal cell proliferation contributes to spinal cord growth,” *J. Comp. Neurol.*, 2012.
[106] E. M. Rodríguez *et al.*, “Hypothalamic tanycytes: A key component of brain-endocrine interaction,” *International Review of Cytology*. 2005.
[107] C. Alfaro-Cervello *et al.*, “The adult macaque spinal cord central canal zone contains proliferative cells and closely resembles the human,” *J. Comp. Neurol.*, 2014.
[108] K. P. Storer, J. Toh, M. A. Stoodley, and N. R. Jones, “The central canal of the human spinal cord: A computerised 3-D study,” *J. Anat.*, 1998.
[109] A. Sakakibara, E. Aoki, Y. Hashizume, N. Mori, and A. Nakayama, “Distribution of nestin and other stem cell-related molecules in developing and diseased human spinal cord,” *Pathol. Int.*, 2007.
[110] H. B. Sarnat, “Role of human fetal ependyma,” *Pediatric Neurology*. 1992.
[111] D. Garcia-Ovejero *et al.*, “The ependymal region of the adult human spinal cord differs from other species and shows ependymoma-like features,” *Brain*, 2015.
[112] C. Dromard *et al.*, “Adult human spinal cord harbors neural precursor cells that generate neurons

and glial cells in vitro," *J. Neurosci. Res.*, 2008.
[113] A. Quiñones-Hinojosa *et al.*, "Cellular composition and cytoarchitecture of the adult human subventricular zone: A niche of neural stem cells," *J. Comp. Neurol.*, 2006.
[114] S. Gil-Perotin, M. Duran-Moreno, S. Belzunegui, M. R. Luquin, and J. M. Garcia-Verdugo, "Ultrastructure of the subventricular zone in Macaca fascicularis and evidence of a mouse-like migratory stream," *J. Comp. Neurol.*, 2009.
[115] M. Sawada *et al.*, "Sensory Input Regulates Spatial and Subtype-Specific Patterns of Neuronal Turnover in the Adult Olfactory Bulb," *J. Neurosci.*, 2011.
[116] Y. Nakayama and K. Kohno, "Number and polarity of the ependymal cilia in the central canal of some vertebrates," *J. Neurocytol.*, 1974.
[117] C. G. Becker, T. Becker, and J. P. Hugnot, "The spinal ependymal zone as a source of endogenous repair cells across vertebrates," *Progress in Neurobiology*. 2018.
[118] R. C. D. Duran *et al.*, "The systematic analysis of coding and long non-coding RNAs in the sub-chronic and chronic stages of spinal cord injury," *Sci. Rep.*, 2017.
[119] A. M. Klein *et al.*, "Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells," *Cell*, 2015.
[120] N. Habib *et al.*, "Div-Seq: Single-nucleus RNA-Seq reveals dynamics of rare adult newborn neurons," *Science (80-.).*, 2016.
[121] X. Fan *et al.*, "Single-cell RNA-seq transcriptome analysis of linear and circular RNAs in mouse preimplantation embryos," *Genome Biol.*, 2015.
[122] P. Codega *et al.*, "Prospective Identification and Purification of Quiescent Adult Neural Stem Cells from Their In Vivo Niche," *Neuron*, 2014.
[123] B. W. Dulken, D. S. Leeman, S. C. Boutet, K. Hebestreit, and A. Brunet, "Single-Cell Transcriptomic Analysis Defines Heterogeneity and Transcriptional Dynamics in the Adult Neural Stem Cell Lineage," *Cell Rep.*, 2017.
[124] V. Silva-Vargas, E. E. Crouch, and F. Doetsch, "Adult neural stem cells and their niche: A dynamic duo during homeostasis, regeneration, and aging," *Current Opinion in Neurobiology*. 2013.
[125] Z. Chaker, P. Codega, and F. Doetsch, "A mosaic world: puzzles revealed by adult neural stem cell heterogeneity," *Wiley Interdisciplinary Reviews: Developmental Biology*. 2016.
[126] I. Imayoshi, M. Sakamoto, and R. Kageyama, "Genetic methods to identify and manipulate newly born neurons in the adult brain," *Frontiers in Neuroscience*. 2011.
[127] E. Llorens-Bobadilla, S. Zhao, A. Baser, G. Saiz-Castro, K. Zwadlo, and A. Martin-Villalba, "Single-Cell Transcriptomics Reveals a Population of Dormant Neural Stem Cells that Become Activated upon Brain Injury," *Cell Stem Cell*, 2015.
[128] A. D. Friedman, "Runx1, c-Myb, and C/EBPα couple differentiation to proliferation or growth arrest during hematopoiesis," *Journal of Cellular Biochemistry*. 2002.
[129] J. T. Gonçalves, S. T. Schafer, and F. H. Gage, "Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior," *Cell*. 2016.
[130] J. Shin *et al.*, "Single-Cell RNA-Seq with Waterfall Reveals Molecular Cascades underlying Adult Neurogenesis," *Cell Stem Cell*, 2015.
[131] T. L. Walker *et al.*, "Prominin-1 Allows Prospective Isolation of Neural Stem Cells from the Adult Murine Hippocampus," *J. Neurosci.*, 2013.
[132] H. Hagihara, K. Ohira, K. Toyama, and T. Miyakawa, "Expression of the AMPA Receptor Subunits GluR1 and GluR2 is Associated with Granule Cell Maturation in the Dentate Gyrus," *Front. Neurogenes.*, 2011.
[133] B. Artegiani, A. Lyubimova, M. Muraro, J. H. van Es, A. van Oudenaarden, and H. Clevers, "A Single-Cell RNA Sequencing Study Reveals Cellular and Molecular Dynamics of the

Hippocampal Neurogenic Niche," *Cell Rep.*, 2017.
[134] D. A. Berg, A. M. Bond, G. Ming, and H. Song, "Radial glial cells in the adult dentate gyrus: what are they and where do they come from?," *F1000Research*, 2018.
[135] D. T. Grimes, C. W. Boswell, N. F. C. Morante, R. M. Henkelman, R. D. Burdine, and B. Ciruna, "Zebrafish models of idiopathic scoliosis link cerebrospinal fluid flow defects to spine curvature," *Science (80-.).*, 2016.
[136] A. Ribeiro, J. F. Monteiro, A. C. Certal, A. M. Cristovão, and L. Saúde, "Foxj1a is expressed in ependymal precursors, controls central canal position and is activated in new ependymal cells during regeneration in zebrafish," *Open Biol.*, 2017.
[137] S. P. Hui, T. C. Nag, and S. Ghosh, "Characterization of Proliferating Neural Progenitors after Spinal Cord Injury in Adult Zebrafish," *PLoS One*, 2015.
[138] A. Reichenbach and H. Wolburg, "Astrocytes and Ependymal Glia," in *Neuroglia*, 2013.
[139] V. Kuscha, S. L. Frazer, T. B. Dias, M. Hibi, T. Becker, and C. G. Becker, "Lesion-induced generation of interneuron cell types in specific dorsoventral domains in the spinal cord of adult zebrafish," *J. Comp. Neurol.*, 2012.
[140] V. Kuscha, A. Barreiro-Iglesias, C. G. Becker, and T. Becker, "Plasticity of tyrosine hydroxylase and serotonergic systems in the regenerating spinal cord of adult zebrafish," *J. Comp. Neurol.*, 2012.
[141] A. J. Mothe and C. H. Tator, "Proliferation, migration, and differentiation of endogenous ependymal region stem/progenitor cells following minimal spinal cord injury in the adult rat," *Neuroscience*, 2005.
[142] S. Khazanov *et al.*, "Floor plate descendants in the ependyma of the adult mouse Central Nervous System," *Int. J. Dev. Biol.*, 2017.
[143] J. Hugnot, "The spinal cord ependymal region: A stem cell niche in the caudal central nervous system," *FEBS J.*, 2014.
[144] R. Fiorelli, A. Cebrian-Silla, J. M. Garcia-Verdugo, and O. Raineteau, "The adult spinal cord harbors a population of GFAP-positive progenitors with limited self-renewal potential," *Glia*, 2013.
[145] A. Orts-Del'Immagine *et al.*, "Postnatal maturation of mouse medullo-spinal cerebrospinal fluid-contacting neurons," *Neuroscience*, 2017.
[146] Y. L. Petracca *et al.*, "The late and dual origin of cerebrospinal fluid-contacting neurons in the mouse spinal cord," *Development*, 2016.
[147] A. L. Huang *et al.*, "The cells and logic for mammalian sour taste detection," *Nature*, 2006.
[148] J. Alfonso, C. Le Magueresse, A. Zuccotti, K. Khodosevich, and H. Monyer, "Diazepam binding inhibitor promotes progenitor proliferation in the postnatal SVZ by reducing GABA signaling," *Cell Stem Cell*, 2012.
[149] A. Pontes, Y. Zhang, and W. Hu, "Novel functions of GABA signaling in adult neurogenesis," *Frontiers in Biology*. 2013.
[150] D. Garcia-Ovejero, A. Arevalo-Martin, B. Paniagua-Torija, Y. Sierra-Palomares, and E. Molina-Holgado, "A cell population that strongly expresses the CB1 cannabinoid receptor in the ependyma of the rat spinal cord," *J. Comp. Neurol.*, 2013.
[151] T. Cawsey, J. Duflou, C. S. Weickert, and C. A. Gorrie, "Nestin-Positive Ependymal Cells Are Increased in the Human Spinal Cord after Traumatic Central Nervous System Injury," *J. Neurotrauma*, 2015.
[152] D. A. Lim and A. Alvarez-Buylla, "The adult ventricular–subventricular zone (V-SVZ) and olfactory bulb (OB) neurogenesis," *Cold Spring Harb. Perspect. Biol.*, 2016.
[153] J. C. Sabourin *et al.*, "A mesenchymal-like ZEB1+ niche harbors dorsal radial glial fibrillary acidic protein-positive stem cells in the spinal cord," *Stem Cells*, 2009.

[154] C. V. Pfenninger, C. Steinhoff, F. Hertwig, and U. A. Nuber, “Prospectively isolated CD133/CD24-positive ependymal cells from the adult spinal cord and lateral ventricle wall differ in their long-term in vitro self-renewal and in vivo gene expression,” *Glia*, 2011.
[155] W. Xu, N. Sachewsky, A. Azimi, M. Hung, A. Gappasov, and C. M. Morshead, “Myelin Basic Protein Regulates Primitive and Definitive Neural Stem Cell Proliferation from the Adult Spinal Cord,” *Stem Cells*, 2017.
[156] X. Li, E. M. Floriddia, K. Toskas, K. J. L. Fernandes, N. Guérout, and F. Barnabé-Heider, “Regenerative Potential of Ependymal Cells for Spinal Cord Injuries Over Time,” *EBioMedicine*, 2016.
[157] J.-P. Hugnot, “The Spinal Cord Neural Stem Cell Niche,” in *Neural Stem Cells and Therapy*, 2012.
[158] C. Göritz, D. O. Dias, N. Tomilin, M. Barbacid, O. Shupliakov, and J. Frisén, “A pericyte origin of spinal cord scar tissue,” *Science (80-.).*, 2011.
[159] L. C. Fuentealba *et al.*, “Embryonic Origin of Postnatal Neural Stem Cells,” *Cell*, 2015.
[160] R. Daneman, D. Agalliu, L. Zhou, F. Kuhnert, C. J. Kuo, and B. A. Barres, “Wnt/ -catenin signaling is required for CNS, but not non-CNS, angiogenesis,” *Proc. Natl. Acad. Sci.*, 2009.
[161] C. E. Scott *et al.*, “SOX9 induces and maintains neural stem cells,” *Nat. Neurosci.*, 2010.
[162] D. A. Turner *et al.*, “Wnt/ -catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells,” *Development*, 2014.
[163] A. Chenn and C. A. Walsh, “Regulation of cerebral cortical size by control of cell cycle exit in neural precursors,” *Science (80-.).*, 2002.
[164] D. Zechner *et al.*, “β-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system,” *Dev. Biol.*, 2003.
[165] A. W. Helms and J. E. Johnson, “Specification of dorsal spinal cord interneurons,” *Current Opinion in Neurobiology*. 2003.
[166] M. Kléber and L. Sommer, “Wnt signaling and the regulation of stem cell function,” *Current Opinion in Cell Biology*. 2004.
[167] S. Megason and A. McMahon, “A mitogen gradient of dorsal midline Wnts organizes growth in the CNS,” *Development*, 2002.
[168] F. Ille *et al.*, “Wnt/BMP signal integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord,” *Dev. Biol.*, 2007.
[169] F. Ulloa and J. Briscoe, “Morphogens and the control of cell proliferation and patterning in the spinal cord,” *Cell Cycle*. 2007.
[170] D. Zechner *et al.*, “Bmp and Wnt/β-catenin signals control expression of the transcription factor Olig3 and the specification of spinal cord neurons,” *Dev. Biol.*, 2007.
[171] A. W. Helms, “Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons,” *Development*, 2005.
[172] T. Müller, K. Anlag, H. Wildner, S. Britsch, M. Treier, and C. Birchmeier, “The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord,” *Genes Dev.*, 2005.
[173] N. A. Bermingham *et al.*, “Proprioceptor pathway development is dependent on MATH1,” *Neuron*, 2001.
[174] K. Gowan *et al.*, “Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons,” *Neuron*, 2001.
[175] L. Wine-Lee, “Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord,” *Development*, 2004.
[176] V. V. Chizhikov and K. J. Millen, “Roof plate-dependent patterning of the vertebrate dorsal central nervous system,” *Dev. Biol.*, 2005.

[177] Y. Muroyama, M. Fujihara, M. Ikeya, H. Kondoh, and S. Takada, "Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord," *Genes Dev.*, 2002.
[178] B. Zhuang and S. Sockanathan, "Dorsal-ventral patterning: A view from the top," *Current Opinion in Neurobiology*. 2006.
[179] K. Azim *et al.*, "Persistent wnt/β-catenin signaling determines dorsalization of the postnatal subventricular zone and neural stem cell specification into oligodendrocytes and glutamatergic neurons," *Stem Cells*, 2014.
[180] N. Masahira *et al.*, "Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells," *Dev. Biol.*, 2006.
[181] L. Xing, T. Anbarchian, J. M. Tsai, G. W. Plant, and R. Nusse, "Wnt/β-catenin signaling regulates ependymal cell development and adult homeostasis," *Proc. Natl. Acad. Sci.*, 2018.
[182] J. R. Timmer, C. Wang, and L. Niswander, "BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors.," *Development*, 2002.
[183] A. Liu and L. A. Niswander, "Bone morphogenetic protein signalling and vertebrate nervous system development," *Nature Reviews Neuroscience*. 2005.
[184] S. Tozer, G. Le Dreau, E. Marti, and J. Briscoe, "Temporal control of BMP signalling determines neuronal subtype identity in the dorsal neural tube," *Development*, 2013.
[185] C. Ramos and B. Robert, "msh/Msx gene family in neural development," *Trends in Genetics*. 2005.
[186] N. Duval *et al.*, "Msx1 and Msx2 act as essential activators of Atoh1 expression in the murine spinal cord," *Development*, 2014.
[187] T. M. Jessell, "Neuronal specification in the spinal cord: Inductive signals and transcriptional codes," *Nature Reviews Genetics*. 2000.
[188] Q. Lei, "Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord," *Development*, 2004.
[189] M. P. Matise, "Molecular genetic control of cell patterning and fate determination in the developing ventral spinal cord," *Wiley Interdisciplinary Reviews: Developmental Biology*. 2013.
[190] M. Sander *et al.*, "Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates," *Genes Dev.*, 2000.
[191] A. Kicheva *et al.*, "Coordination of progenitor specification and growth in mouse and chick spinal cord," *Science (80-.).*, 2014.
[192] J. Briscoe, A. Pierani, T. M. Jessell, and J. Ericson, "A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube," *Cell*, 2000.
[193] M. Barzi, D. Kostrz, A. Menendez, and S. Pons, "Sonic hedgehog-induced proliferation requires specific gα inhibitory proteins," *J. Biol. Chem.*, 2011.
[194] D. Stamataki, F. Ulloa, S. V. Tsoni, A. Mynett, and J. Briscoe, "A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube," *Genes Dev.*, 2005.
[195] E. Dessaud, A. P. McMahon, and J. Briscoe, "Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network," *Development*, 2008.
[196] F. Bangs and K. V. Anderson, "Primary cilia and Mammalian Hedgehog signaling," *Cold Spring Harb. Perspect. Biol.*, 2017.
[197] S. C. Goetz and K. V. Anderson, "The primary cilium: A signalling centre during vertebrate development," *Nature Reviews Genetics*. 2010.
[198] R. Rohatgi, L. Milenkovic, and M. P. Scott, "Patched1 regulates hedgehog signaling at the primary cilium," *Science (80-.).*, 2007.
[199] M. Cohen *et al.*, "Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms,"

Nat. Commun., 2015.
[200] J. Briscoe and S. Small, "Morphogen rules: design principles of gradient-mediated embryo patterning," *Development*, 2015.
[201] A. Iulianella, D. Sakai, H. Kurosaka, and P. A. Trainor, "Ventral neural patterning in the absence of a Shh activity gradient from the floorplate," *Dev. Dyn.*, 2018.
[202] E. Dessaud *et al.*, "Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog," *PLoS Biol.*, 2010.
[203] V. Ribes and J. Briscoe, "Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback.," *Cold Spring Harbor perspectives in biology*. 2009.
[204] J. E. Burda and M. V. Sofroniew, "Reactive gliosis and the multicellular response to CNS damage and disease," *Neuron*. 2014.
[205] C. S. Ahuja *et al.*, "Traumatic spinal cord injury - Repair and regeneration," *Clin. Neurosurg.*, 2017.
[206] O. N. Hausmann, "Post-traumatic inflammation following spinal cord injury," *Spinal Cord*. 2003.
[207] A. Alizadeh, S. M. Dyck, and S. Karimi-Abdolrezaee, "Myelin damage and repair in pathologic CNS: challenges and prospects," *Front. Mol. Neurosci.*, 2015.
[208] M. T. Filbin, "Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS," *Nat. Rev. Neurosci.*, 2003.
[209] R. J. M. Franklin and C. Ffrench-Constant, "Regenerating CNS myelin - From mechanisms to experimental medicines," *Nature Reviews Neuroscience*. 2017.
[210] C. Soderblom *et al.*, "Perivascular Fibroblasts Form the Fibrotic Scar after Contusive Spinal Cord Injury," *J. Neurosci.*, 2013.
[211] T. M. O'Shea, J. E. Burda, and M. V. Sofroniew, "Cell biology of spinal cord injury and repair," *Journal of Clinical Investigation*. 2017.
[212] M. A. Anderson *et al.*, "Astrocyte scar formation AIDS central nervous system axon regeneration," *Nature*, 2016.
[213] S. Bardehle *et al.*, "Live imaging of astrocyte responses to acute injury reveals selective juxtavascular proliferation," *Nat. Neurosci.*, 2013.
[214] I. B. Wanner *et al.*, "Glial Scar Borders Are Formed by Newly Proliferated, Elongated Astrocytes That Interact to Corral Inflammatory and Fibrotic Cells via STAT3-Dependent Mechanisms after Spinal Cord Injury," *J. Neurosci.*, 2013.
[215] M. Tsintou, K. Dalamagkas, and A. M. Seifalian, "Advances in regenerative therapies for spinal cord injury: A biomaterials approach," *Neural Regen. Res.*, 2015.
[216] A. Barreiro-Iglesias *et al.*, "Serotonin Promotes Development and Regeneration of Spinal Motor Neurons in Zebrafish," *Cell Rep.*, 2015.
[217] M. M. Reimer *et al.*, "Dopamine from the Brain Promotes Spinal Motor Neuron Generation during Development and Adult Regeneration," *Dev. Cell*, 2013.
[218] K. Ogai, K. Nakatani, S. Hisano, K. Sugitani, Y. Koriyama, and S. Kato, "Function of Sox2 in ependymal cells of lesioned spinal cords in adult zebrafish," *Neurosci. Res.*, 2014.
[219] M. H. Mokalled, C. Patra, A. L. Dickson, T. Endo, D. Y. R. Stainier, and K. D. Poss, "Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish," *Science (80-.).*, 2016.
[220] V. M. Tysseling *et al.*, "SDF1 in the dorsal corticospinal tract promotes CXCR4+ cell migration after spinal cord injury," *J. Neuroinflammation*, 2011.
[221] I. Pineau and S. Lacroix, "Proinflammatory cytokine synthesis in the injured mouse spinal cord: Multiphasic expression pattern and identification of the cell types involved," *J. Comp. Neurol.*,

2007.
[222] J. C. Gensel and B. Zhang, “Macrophage activation and its role in repair and pathology after spinal cord injury,” *Brain Res.*, 2015.
[223] B. Zhang and J. C. Gensel, “Is neuroinflammation in the injured spinal cord different than in the brain? Examining intrinsic differences between the brain and spinal cord,” *Experimental Neurology*. 2014.
[224] S. David and A. Kroner, “Repertoire of microglial and macrophage responses after spinal cord injury,” *Nature Reviews Neuroscience*. 2011.
[225] F. M. Bareyre and M. E. Schwab, “Inflammation, degeneration and regeneration in the injured spinal cord: Insights from DNA microarrays,” *Trends in Neurosciences*. 2003.
[226] D. J. Donnelly and P. G. Popovich, “Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury,” *Exp. Neurol.*, 2007.
[227] K. A. Kigerl, J. C. Gensel, D. P. Ankeny, J. K. Alexander, D. J. Donnelly, and P. G. Popovich, “Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord,” *J. Neurosci.*, 2009.
[228] J. C. Furlan, M. B. Bracken, and M. G. Fehlings, “Is age a key determinant of mortality and neurological outcome after acute traumatic spinal cord injury?,” *Neurobiol. Aging*, 2010.
[229] M. Bouab, G. N. Paliouras, A. Aumont, K. Forest-Bérard, and K. J. L. Fernandes, “Aging of the subventricular zone neural stem cell niche: Evidence for quiescence-associated changes between early and mid-adulthood,” *Neuroscience*, 2011.
[230] J. C. Conover and B. A. Shook, “Aging of the subventricular zone neural stem cell niche.,” *Aging Dis*, 2011.
[231] V. Capilla-Gonzalez, A. Cebrian-Silla, H. Guerrero-Cazares, J. M. Garcia-Verdugo, and A. Quiñones-Hinojosa, “Age-related changes in astrocytic and ependymal cells of the subventricular zone,” *Glia*, 2014.
[232] C. G. Geoffroy, B. J. Hilton, W. Tetzlaff, and B. Zheng, “Evidence for an Age-Dependent Decline in Axon Regeneration in the Adult Mammalian Central Nervous System,” *Cell Rep.*, 2016.
[233] H. Sabelström *et al.*, “Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice,” *Science (80-.).*, 2013.
[234] F. Barnabé-Heider and J. Frisén, “Stem cells for spinal cord repair,” *Cell Stem Cell*. 2008.
[235] V. Neirinckx, C. Coste, R. Franzen, A. Gothot, B. Rogister, and S. Wislet, “Neutrophil contribution to spinal cord injury and repair,” *Journal of Neuroinflammation*. 2014.
[236] X. C. Li, C. F. Zhong, G. Bin Deng, R. W. Liang, and C. M. Huang, “Efficacy and safety of bone marrow-derived cell transplantation for spinal cord injury: A systematic review and meta-analysis of clinical trials,” *Clin. Transplant.*, 2015.
[237] D. Jarocha, O. Milczarek, A. Wedrychowicz, S. Kwiatkowski, and M. Majka, “Continuous improvement after multiple mesenchymal stem cell transplantations in a patient with complete spinal cord injury,” *Cell Transplant.*, 2015.
[238] H. Iwai *et al.*, “Allogeneic Neural Stem/Progenitor Cells Derived From Embryonic Stem Cells Promote Functional Recovery After Transplantation Into Injured Spinal Cord of Nonhuman Primates,” *Stem Cells Transl. Med.*, 2015.
[239] A. H. All *et al.*, “Early intervention for spinal cord injury with human induced pluripotent stem cells oligodendrocyte progenitors,” *PLoS One*, 2015.
[240] H. Kumamaru *et al.*, “Generation and post-injury integration of human spinal cord neural stem cells,” *Nat. Methods*, 2018.
[241] N. M. P. King and J. Perrin, “Ethical issues in stem cell research and therapy,” *Stem Cell Research and Therapy*. 2014.

[242] C. P. Hofstetter *et al.*, "Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome," *Nat. Neurosci.*, 2005.
[243] H. Yang, B. R. He, and D. J. Hao, "Biological Roles of Olfactory Ensheathing Cells in Facilitating Neural Regeneration: A Systematic Review," *Molecular Neurobiology*. 2014.
[244] A. Honoré *et al.*, "Isolation, characterization, and genetic profiling of subpopulations of olfactory ensheathing cells from the olfactory bulb," *Glia*, 2012.
[245] A. P. Pêgo *et al.*, "Regenerative medicine for the treatment of spinal cord injury: More than just promises?," *J. Cell. Mol. Med.*, 2012.
[246] J. Li and G. Lepski, "Cell transplantation for spinal cord injury: A systematic review," *BioMed Research International*. 2013.
[247] P. Assinck, G. J. Duncan, B. J. Hilton, J. R. Plemel, and W. Tetzlaff, "Cell transplantation therapy for spinal cord injury," *Nature Neuroscience*. 2017.
[248] I. Vismara, S. Papa, F. Rossi, G. Forloni, and P. Veglianese, "Current Options for Cell Therapy in Spinal Cord Injury," *Trends in Molecular Medicine*. 2017.
[249] H. Nakajima *et al.*, "Transplantation of Mesenchymal Stem Cells Promotes an Alternative Pathway of Macrophage Activation and Functional Recovery after Spinal Cord Injury," *J. Neurotrauma*, 2012.
[250] N. Weishaupt, A. Blesch, and K. Fouad, "BDNF: The career of a multifaceted neurotrophin in spinal cord injury," *Experimental Neurology*. 2012.
[251] D. H. Hwang *et al.*, "Combination of multifaceted strategies to maximize the therapeutic benefits of neural stem cell transplantation for spinal cord repair," *Cell Transplant.*, 2011.
[252] E. J. Benner *et al.*, "Protective astrogenesis from the SVZ niche after injury is controlled by Notch modulator Thbs4," *Nature*, 2013.
[253] G. Yiu and Z. He, "Glial inhibition of CNS axon regeneration," *Nature Reviews Neuroscience*. 2006.
[254] J. M. Cregg, M. A. DePaul, A. R. Filous, B. T. Lang, A. Tran, and J. Silver, "Functional regeneration beyond the glial scar," *Experimental Neurology*. 2014.
[255] A. Manuscript, "Don't Fence me in: Harnessing the beneficial roles of astrocytes for spinal cord repair," *Changes*, 2012.
[256] M. Pekny, U. Wilhelmsson, and M. Pekna, "The dual role of astrocyte activation and reactive gliosis," *Neuroscience Letters*. 2014.
[257] M. Ronaghi, S. Erceg, V. Moreno-Manzano, and M. Stojkovic, "Challenges of stem cell therapy for spinal cord injury: Human embryonic stem cells, endogenous neural stem cells, or induced pluripotent stem cells?," *Stem Cells*. 2010.

Abstract: Anamniotes can regenerate a spinal cord after lesion due to endogenous stem/ progenitor cells activation. Investigating the presence and properties of such cells in mammals one could possibly harness those cells toward regeneration including neurons. We conducted RNA profiling to compare human vs mouse stem cell niche and lesioned vs non lesioned mouse spinal cord stem cell niche. At least 5 cell types were revealed and here a new dorsal cell type expressing Msx1 and Id4 transcription factors was identified. Our data indicated that the adult spinal cord niche in mouse and human is a mosaic of cells with different developmental origin. Though after lesion, niche stem cells are activated, through pathways favoring astrocyte generation. Glial-neuronal interactions supporting and keeping neurons intact can influence neurodegenerative diseases. One of these glial cells is the perineuronal satellite cells. PNCs are are neither oligodendrocyte progenitor cells nor myelinating oligodendrocytes and tightly associated to the soma of large neurons. Our data suggest that PNCs could be implicated in neurodegenerative diseases such as multiple sclerosis and amyotrophic lateral sclerosis due to their interaction with motor neurons.
Keywords: Spinal cord, Stem cells, Niche, PNCs

Résume: Les anamniotes peuvent régénérer la moelle épinière après une lésion en raison de l'activation de cellules souches / progénitrices endogènes. En recherchant la présence et les propriétés de telles cellules chez les mammifères, on pourrait exploiter ces cellules vers la régénération, y compris les neurones. Nous avons procédé au profilage de l'ARN afin de comparer la niche de cellules souches humaine vs souris et la niche de cellules souches de la moelle épinière de souris non lésées. Au moins 5 types de cellules ont été révélés et un nouveau type de cellules dorsales exprimant les facteurs de transcription Msx1 et Id4 a été identifié. Nos données ont indiqué que la niche de la moelle épinière chez la souris et chez l'homme est une mosaïque de cellules d'origine développementale différente. Après la lésion, les cellules souches de niche sont activées par des voies favorisant la génération d'astrocytes. Les interactions gliales-neuronales soutenant et maintenant les neurones intacts peuvent influencer les maladies neurodégénératives. L'une de ces cellules gliales est la cellule satellite périneuronale. Les PNC ne sont ni des cellules progénitrices des oligodendrocytes ni des oligodendrocytes myélinisants et sont étroitement associés au soma de gros neurones. Nos données suggèrent que les PNC pourraient être impliquées dans les maladies neurodégénératives telles que la sclérose en plaques et la sclérose latérale amyotrophique en raison de leur interaction avec les motoneurones.
Mots clés: Moelle épinière, Cellules souches, Niche, PNCs.