Transcriptional regulation and cellular strategies in neuroregeneration

Transcriptionele regulatie en cellulaire strategieën in neuroregeneratie (met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

Introduction

Embryonic neural development: from neuroepithelium to radial glia

In 1899, Santiago Ramón y Cayal comprehensively described in his anatomical studies the structure and organization of the nervous system (Kandel *et al.,* 2000). Ever since, countless studies have been focused on understanding the development of the nervous system that he described. Development of the nervous system depends on -time and -space regulated gene expression. These molecular programs and epigenetic mechanisms are regulated by signals arising from the embryo and from the external environment. Many important advances have arisen from the study of simple organisms such as *Drosophila melanogaster* or *Caenorbitis elegans*, which are also easily genetically modifiable (Kandel *et al.,* 2000). In vertebrates, the central nervous system (CNS), which comprises the brain and the spinal cord, is one of the first systems to begin its development during embryogenesis only reaching completion after birth. At the blastocyst stage the embryo is an oval structure formed from an upper layer (epiblast cells) and a lower layer (hypoblast cells). During gastrulation, the epiblast cells differentiate into the three germ layers that will form all the different structures of the developing embryo, namely the endoderm, the mesoderm and the ectoderm. The ectoderm gives rise to the neural plate, formed by neuroepithelial stem cells, which are considered the first specialized precursors of neural tissues. Next, the neural plate folds into the neural tube, a hollow tube structure. When the neural tube is complete, the neural progenitor cells are localized as a single layer at the hollow center of the neural tube. As the development progresses the shape of the neural tube changes giving origin to the ventricular system of the brain. Neuroepithelial cells are located in the region that will eventually become the ventricles, and defined as the ventricular zone (VZ) (Stiles and Jernigan, 2010). Neural progenitors located in the rostral region of the neural tube will form brain and the caudally located neural progenitors will form the spinal cord. Subsequently, the neural tube expands into three primary brain vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon). These vesicles will then subdivide into five vesicles forming the telencephalon and the diencephalon (deriving from the prosencephalon), the metencephalon and the myelencephalon (arising from the rhombencephalon) and the mesencephalon (Kandel *et al.,* 2000). These regions will eventually give origin to the six major regions of the brain. (Kandel *et al.,* 2000).

A well-studied example of embryonic neurogenesis is the developing neocortex. Around embryonic day (E) 9–10 in the mouse, neuroepithelial cells in this region start acquiring features associated the radial glia (RG) cells that are considered to be embryonic neural stem cells (NSCs). These cells remain in contact with both the pial and the ventricular surface, with their cell bodies being retained within the VZ. During the thickening of the cortex the RG cells begin to express astroglial markers such as the glutamate transporter (GLAST), brain lipid-binding protein (BLBP), and Tenascin C (TN-C) (Campbell and Gotz, 2002). Moreover they also express a number of intermediate filament proteins including nestin and vimentin (Mori *et al.,* 2005). RG cells show apical-basal polarity and line the lateral ventricles, from which, through interkinetic nuclear migration, give origin to a pseudostratified epithelium

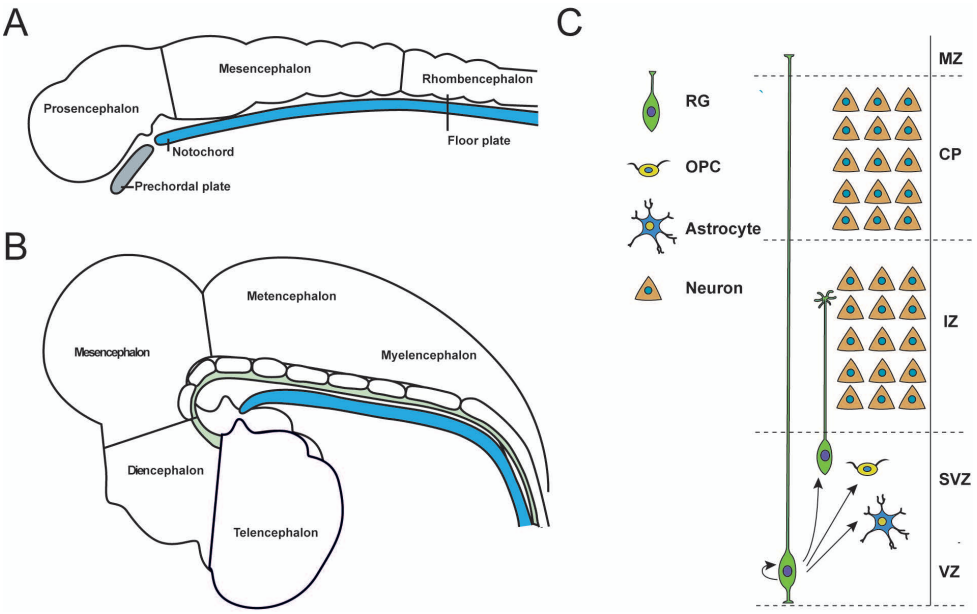

Figure 1: Schematic representation of the developing vertebrate neural tube and neocortex.

(**A**) Representation of the three primary brain vesicles. (**B**) Representation of the five vesicles forming the telencephalon and the diencephalon), the metencephalon and the myelencephalon and the mesencephalon. (**C**) Representation of the developing cortex. MZ: marginal zone; CP: cortical plate; IZ: intermediate zone; OPC: oligodendrocyte precursor cell.

within the VZ (Kriegstein and Alvarez-Buylla, 2009). RG cells are considered to be the neural stem cells that subsequently generate differentiated neurons and glial cells (astrocytes and oligodendrocytes) during development and in the postnatal brain. In 1992, Reynold and Weiss reported for the first time the presence of NSCs in the adult brain (Reynolds and Weiss, 1992). In fact, NSCs are also found in restricted regions of postnatal and adult brains where they continue to produce neurons and glial cells. New neurons are generated within the subventricular zone (SVZ) in the lateral ventricles and in the adult hippocampus in the subgranular layer (SGL) of the dentate gyrus (DG). These NSCs have characteristics similar to RG cells (Kriegstein and Alvarez-Buylla, 2009). Moreover, NSCs participate in regenerating the brain in response to injury, however, the regenerative potential of the brain remains poor (Fuentealba et al., 2012; Miller and Gauthier-Fisher, 2009). To this end, it is crucial to investigate the molecular mechanisms regulating NSC biology in order to develop better therapeutic strategies to repair the damaged CNS.

Major signaling pathways regulating NSC function

A number of well-defined temporal and spatial signals determine the proliferation, self-renewal capacity and differentiation of NSCs. Here three major signaling pathways will be highlighted as central in regulating NSCs: canonical Wnt, Sonic hedgehog (Shh) and Notch (see **Figure 2**).

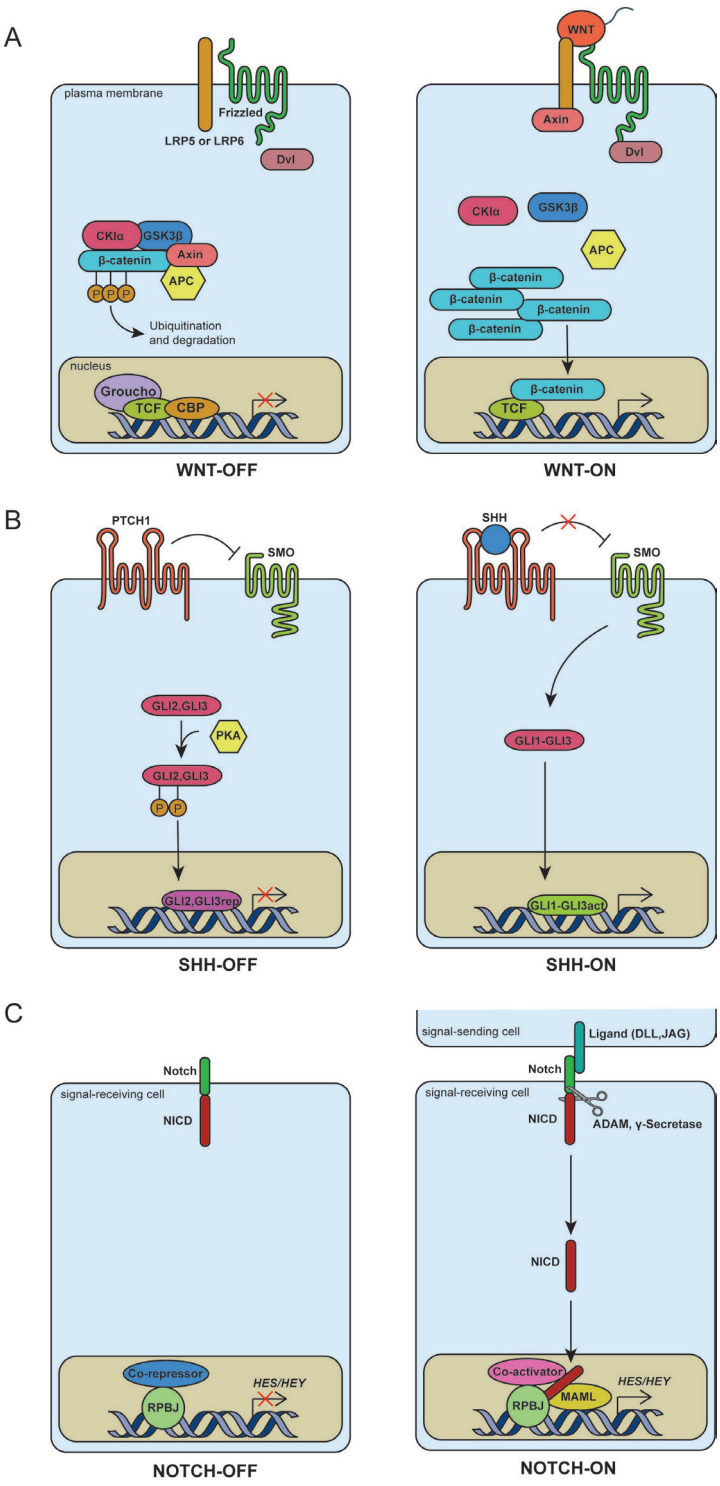

Figure 2: Schematic representation of Wnt, Shh and Notch pathways.

(**A**) In absence of WNT (WNT-OFF), β-catenin is targeted for degradation by the destruction complex, formed by casein kinase I α (CKIα), glycogen synthase kinase 3 β (GSK3β), Axin and adenomatous polyposis coli (APC). The transcriptional repressor Groucho is bound to the transcription factors TCF/LEF and CBP, thereby inhibiting transcription of target genes. In presence of WNT (WNT-ON), dishevelled (Dvl) binds to the intracellular domain of the frizzled transmembrane receptor and to Axin, inhibiting its activity. Hence, β-catenin is not degraded and accumulates in the cytoplasm, thereby entering the nucleus and, by binding with T-cell factor/lymphoid enhancer factor (TCF/LEF), inducing expression of target genes. (**B**) In absence of SHH (SHH-OFF), the Patched (PTCH1) inhibits Smoothened (SMO) activity allowing the protein kinase A (PKA)-mediated phosphorylation and truncation of GLI2 and GLI3. GLI2repressor(rep) and GLI3rep form enter the nucleus and inhibit gene transcription. In presence of SHH (SHH-ON), SMO is no longer inhibited by PTCH1, activating GLI. GLI1activator(act)-GLI2act form enter the nucleus and induce target gene transcription. (**C**) In absence of Notch-ligands (NOTCH-OFF), recombination signal binding protein for immunoglobulin kappa J region (RPBJ) interacts with co-repressors in the nucleus and inhibits target gene transcription. Upon ligand presentation by signaling-sending cell, Notch-intracellular domain (NICD) is cleaved in the signaling-receiving cell, enters the nucleus and, by interacting with RPBJ, mastermind-like (MAML) and other co-activators, induces transcription of target genes such as *HEY* and *HES*.

Wnt pathway

Wnt is a soluble ligand that when binding to the receptors Frizzled and LRP induces a series of intracellular changes that will result in the translocation of β-catenin to the nucleus, with consequent activation of the expression of Wnt target genes, such as Axin2 and Jagged1 (the latter further discussed in this introduction) (Bowman *et al.,* 2013; Chen *et al.,* 2010). In particular, lineage tracing experiments, utilizing a reporter consisting of a tamoxifen-inducible Cre protein from the endogenous Axin2 locus and a Rosa26 membrane tomato/membrane green fluorescent protein reporter system, showed that Axin2 expression marked the neural lineage at different developmental time points in mice. These cells later became functional adult NSCs in the SVZ and DG. Finally, cells in these adult niches were also shown to be Wnt/β-catenin-responsive (Bowman *et al.,* 2013). Taken together, these observations mean that Wnt signaling is active both in adult and embryonic murine neural progenitor. β-catenin is an activating co-factor for the TCF/LEF transcription factors family, and is expressed, although not exclusively, within the SVZ. Depletion of β-catenin in mice resulted in overall size reduction of the brain and spinal cord, accompanied by a loss in progenitor population (Zechner *et al.,* 2003). Conversely expression of a mutant constitutively activated form of β-catenin resulted in increased CNS size and expansion of neuronal progenitor population (Zechner *et al.,* 2003). Furthermore, mice expressing a stabilized form of β-catenin present enlarged brains and increased cerebral cortex, through the induction of proliferation of neural precursor cells (Chenn and Walsh, 2002). These observations suggest that β-catenin promotes neural progenitor proliferation. Consistently, Wnt3a knockout mice exhibited a reduced hippocampus size, resulting from reduction in progenitor proliferation (Lee *et al*., 2000). In contrast with these findings, it has been show that ectopic Wnt3a induced differentiation of intermediate cortical progenitors in a β-catenin-independent manner, meaning that different kinds of Wnt-signalling play a differential role in neural progenitor maintenance and induction of differentiation (Bengoa-Vergniory and Kypta, 2015). Canonical Wnt receptors have also been shown to be important in neurogenesis. Fzd3 knockout mice bear defects in axonal guidance and extensive cell death in the striatum during late embryonic development, probably due to the nearly complete absence of long-range

connections (Wang *et al.,* 2002). Furthermore, LRP6 knockout mice presented hypoplasia of the developing neocortex, due to reduced proliferation in late embryonic development (Zhou *et al.,* 2006). Stabilization of the inhibitor of the Wnt pathway Axin, with the tankirase inhibitor IWR-1 impairs NSC proliferation in neurospheres derived from murine cortex (Chen *et al.,* 2013). Taken together these observations show that Wnt/β-catenin signalling has a pivotal role in promoting NSC proliferation and maintenance.

Shh pathway

Another key pathway regulating NSCs is represented by the Shh pathway. Shh is a secreted protein that drives morphogenesis in several tissues and organs, as the limb, heart, neural tube and cerebellum (Komada, 2012). Shh binding to its receptors Patched-1 and Patched-2 will eventually turn into the activation of the transcription factors from the Gli family (Gli1, -2 and -3) (Villavicencio *et al.,* 2000).

Shh regulates progenitor proliferation of RG and intermediate progenitors (IP) in the dorsal telecephalon, as RG specific Shh KO mice presented reduced proliferation of RG and IP (Tbr2-positive neural progenitor cells) (Shikata *et al.,* 2011). Moreover, genetic deletion of Shh in the early developing dorsal telencephalon by the use of Emx1-Cre strain induced reduction of the telencephalon cause by prolonged cell cycle in neural progenitor/neural stem cell (Komada *et al.,* 2008). These observations suggest that Shh promotes the proliferation of neural stem/progenitor cells during development. Additionally, expression of the downstream Shh effector Gli-1 in Nestin positive NSCs/ neural progenitor induces expansion of neural progenitor and their clonogenic potential both *in vivo* and *in vitro* (Stecca and Ruiz i Altaba, 2009). Depletion of the Shh targets Gli2 and Gli3 in E18.5 cortical tissue reduces primary and secondary murine neurosphere formation. Moreover, Gli2 depletion in NSCs inhibits cell proliferation and the expression of Sox2 and other NSC markers, including Hes1, Hes5 and Notch1 (Ahmed *et al.,* 2009; Takanaga *et al.,* 2008). Taken together these observations indicate that the Shh pathway promotes neural progenitor proliferation.

Notch pathway

The Notch signaling pathway is activated by the binding of Notch ligands with the Notch receptors, such as the Deltra/Serrate/Lag2 (DSL) family (such as Dll1, -2, -3, -4, Jagged1 and Jagged2 in mouse) present on the cell surface of the neighboring cells. Subsequently Notch is cleaved by ADAM (a disintegrin and metalloprotease) metalloprotease and by the cleaving protease γ-secretase (Kopan and Ilagan, 2009). This causes the release of the Notch intracellular domain (NICD) which subsequently enters the nucleus and associates with the DNA-binding protein CSL. Expression of Notch target genes is induced, including the basic-loop-helix (bHLH) transcriptional repressors belonging to the HES/ESR/HEY family (such as Hes1, Hes5 and Hey1) (Kopan and Ilagan, 2009).

Notch has been linked to neural development since its discovery in 1940, when Poulson described how the deletion of a chromosomic region, later described to contain the *Notch* locus, resulted in hypertrophy of the neural tissue at the expense of other epidermal structures in *Drosophila melanogaster* embryos, giving origin to the so-called neurogenic phenotype (Louvi and Artavanis-Tsakonas, 2006). This phenotype can be explained because in absence of Notch-dependent signaling the neighboring cells differentiate into neuroblast, hence inducing hypertrophy of the neural tissue (Louvi and Artavanis-Tsakonas, 2006). Notch signaling has been extensively described as inhibitor of neuronal differentiation. Expression of constitutively active forms of the Notch receptor in the retina of *Xenopus laevis* inhibits neuronal differentiation (Coffman *et al.,* 1993). Similarly, expression of active Notch and in the eye of *D. melanogaster* inhibits neuronal fate, although in a reversible manner: once the neuronal precursors ceased to express the constitutively active Notch, the cells differentiated normally towards the neuronal lineage (Fortini *et al.,* 1993). This suggests that Notch maintains a precursor state until the correct developmental signals occur (Louvi and Artavanis-Tsakonas, 2006). Furthermore, mimicking the activation of Notch by inducing the expression of Hes1 inhibits neuronal differentiation in mice, causing the $Hes1^{+}$ cells to localize in the VZ of the cortex (Ishibashi *et al.,* 1994). Similarly, retroviral-mediated expression of an activated form of Notch 1 at E9.5 inhibits neuronal differentiation by inducing the expressing cells to acquire RG characteristics (Gaiano *et al.,* 2000). Shimojo *et al*. showed that Hes1 expression dynamically oscillates in neural progenitor cells and that Hes1 downregulates the expression of pro-neural genes as Neurogenin2 (Ngn2), a basic-helix-loop-helix transcription factor which induces neuronal differentiation, thereby maintaining a progenitor state (Shimojo *et al.,* 2008). Consistently, in mouse embryonic NSCs, Hes1 and Hes5 levels have been shown to oscillate in opposite direction of the expression of their repressed target Ascl1, thus promoting progenitor proliferation (Imayoshi *et al.,* 2013). The role of Jagged1 (Jag1) in NSC differentiation has been also investigated. In the murine postnatal SVZ, Jag1 acts through Notch1 to promote NSC proliferation (Nyfeler et al., 2005). Ottone and colleagues showed that Jag1 expressed by endothelial cells in the adult SVZ induces quiescence of NSCs, since depletion of Jag1 induces exhaustion of these progenitors (Ottone *et al.,* 2014). Notch is known promote gliogenesis, with the exception of oligodendrocytes (Louvi and Artavanis-Tsakonas, 2006). Activation of Notch signaling in retinal progenitors in zebrafish blocks neuronal differentiation, at the same time leading to excessive and premature gliogenesis (Scheer *et al.,* 2001). Similarly, overexpression of Hes5 in the retinae of postnatal mice promotes gliogenesis and reduces neurogenesis, while deletion of Hes5 reduces differentiation of Müller glia (Hojo et al., 2000). The same is true for Hes1, since its ectopic expression promotes gliogenesis, while the induction of a dominant negative form of Hes1 reduces the number of glial cells (Furukawa et al., 2000). Notch signaling has been shown to inhibit oligodendrocyte differentiation. Expression of Jag1 activates Notch1 in immature oligodendrocytes, thereby preventing their maturation. To allow oligodendrocyte maturation and myelination, Jagged1 has to be downregulated (Wang et al., 1998) while $Notch1^{+/-}$ mice exhibit premature myelination (Givogri *et al.,* 2002). Finally, Hes5 has been shown to inhibit the

expression of the thyroid hormone receptor protein (TRβ1) in oligodendrocyte precursor cells, which is necessary for oligodendrocyte differentiation (Kondo and Raff, 2000).

Taken together these observations indicate that Notch signaling is essential by maintaining the neural progenitor state and inhibiting neuronal differentiation. Moreover, Notch promotes glia differentiation while it specifically inhibits oligodendrocyte maturation.

These aforementioned pathways are finely regulated at different levels. In particular, transcriptional regulation of the expression of the components defining these molecular cascades is an essential way to fine tune NSC biology. Hence the necessity of studying the role of transcription factors in NSCs.

The role of Forkhead box proteins in regulating neurogenesis

Forkhead box (Fox) proteins are a subfamily of the winged helix-turn-helix DNA-binding domain superfamily, a large family of transcription factors involved in the regulation of multiple cellular functions including neurogenesis (Greer and Brunet, 2005; Maiese *et al.,* 2008; Zhao *et al.,* 2007). The canonical forkhead domain, consisting of 3 amino-terminal α-helices, 3 β-sheets, a hydrophobic core and 2 loops, interacts with the phosphate backbone of the target DNA molecule (Genin et al., 2014). The Fox family, which comprises both transcriptional activators and repressors, bind the DNA in correspondence of Fox-recognize element, whose core consensus sequence is 5'-(G/A)(T/C)(A/C)AA(C/T)A-3'. Flanking sequences on both sides of the core sequence are also involved in the formation of a high affinity binding (Lehmann *et al.,* 2003). The other domains of the Fox factors, such as transactivation/transrepression domains, are mostly non-conserved; however it is possible to identify conserved regions and motifs within specific subclasses of the Fox family (**Figure 3**).

Fox proteins interact with several cofactors, for example homodomain proteins and nuclear receptors that modulate their capacity of regulating their target genes. **Table 1** summarizes the literature about the Fox factors involved in neurogenesis.

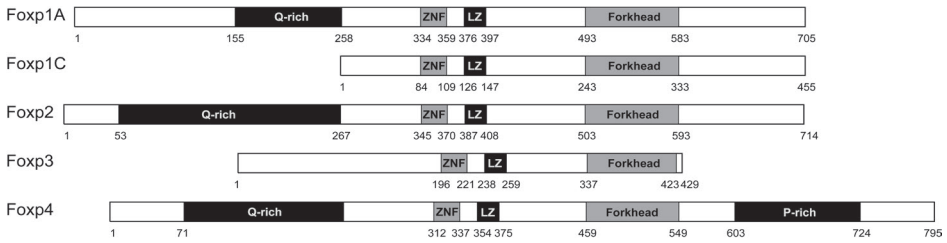

Figure 3: Schematic representation of Foxp transcription factors.

Depiction of the domains of the four members of the Foxp factors and the Foxp1C isoform. Q-rich: glycine-rich domain; ZNF: zinc-finger domain; LZ: Leucine-zipper domain; Forkhead: Forkhead domain; P-rich: proline-rich domain.

Table 1. Fox factors involved in neurogenesis.

	Expression	Role	Reference
FoxO1, FoxO3, FoxO6	NSCs from E12 to E14.	Inhibit proliferation and self-renewal of mouse embryonic NSCs by activating ASPM, Sfrp1/2, Sost	(Hoekman *et al.,* 2006)
FoxO3	NSCs	FoxO3 KO mice have reduced amount of NSCs as FoxO3 prevents differentiation and controls O_2 metabolism. Maintain redox balance and neurogenic potential NSCs. Counteracts ASCL1 to maintain neuorogenic potential	(Renault *et al.,* 2009) (Yeo *et al.,* 2013) (Webb and Brunet, 2013)
FoxO1	NSCs	Represses differentiation by activating Notch pathway	(Kim *et al.,* 2015)
Foxg1	postnatal hippocampus	$Foxg1^{+/-}$ mice have reduced neurogenesis in the DG. Foxg1 inhibits gliogenesis and induces neuronal differentiation in cultured NSCs Maintain NSC self-renewal in the forebrain with Bmi1	(Shen *et al.,* 2006) (Tian *et al.,* 2012) (Brancaccio *et al.,* 2010) (Fasano *et al.,* 2009)
Foxj1	Ependimal cells, olphactory bulb Neuronal progenitors	Foxj1 promotes the differentiation of RG into ependymal cells	(Jacquet *et al.,* 2009)
Foxm1	NSCs	Promotes NSC self-renewal. Loss of Foxm1 correlates with reduced Sox2 expression	(Wang *et al.,* 2011)

FoxP factors

The FoxP family, formed by FoxP1-4 proteins, is a unique subgroup of the Fox family as they contain both a zinc-finger and a leucine-zipper. These domains provide the capacity to homo- or hetero-dimerize in order to bind the DNA, and therefore exert their regulatory activity (Bacon and Rappold, 2012). FoxP1 and FoxP2 have been described to exert a function in the CNS as well as being linked with cognitive disorders (Bacon and Rappold, 2012). Human FOXP2 mutations have been linked to developmental verbal dyspraxia (DVD) (Feuk *et al.,* 2006). In particular, mutations causing DVD affect Foxp2 function, mainly by disrupting nuclear localization and DNA-binding properties (Vernes *et al.,* 2006). Moreover, Foxp2 mutant mice show reduced neurite outgrowth during embryonic development. This is because Foxp2 inhibits genes involved in the development and plasticity of neuronal connections like Nrn1, Nell2 and Cck (Vernes et al., 2011). Finally, FoxP2 has been shown to drive human neuronal differentiation. In fact, neurons expressing FoxP2 showed increased neurite outgrowth and reduced migration. This effect is due to FoxP2 increasing the sensitivity to retinoic acid, know to promote neuronal differentiation (Devanna *et al.,* 2014). FoxP4 function has not been extensively characterized regarding its role in the CNS. However it is expressed in the developing and adult rat forebrain (Takahashi *et al.,* 2008). Moreover, Foxp4 has been described as essential for the arborization of Purkinje cells in the mouse cerebellum (Tam *et al.,* 2011). Finally, it is found co-expressed with Foxp1 and Foxp2 in specialized brain areas that control song production in zebra finch, suggesting these three members might physically interact (Mendoza *et al.,* 2015).

FoxP1 is generally considered to be a transcriptional repressor and has been associated with a wide range of biological functions including development of heart, lung, esophagus, immune system and spinal motor neurons. Moreover the role of Foxp1 in cancer has been extensively characterized (Bacon and Rappold, 2012). Foxp1-knockout mice die at E14.5 of cardiac morphogenesis defects, including outflow tract separation, cushion defects as well as myocardial maturation/ proliferation deregulation (Wang *et al.,* 2004). Different isoforms of murine Foxp1 have been described (see **Figure 3**): Foxp1A (79 kDa), Foxp1B (72 kDa) lacking residues 539-602, Foxp1C (50 kDa) lacking residues 1-250, and Foxp1-ES (80kDa) which has an alternative coding sequence at residues 540-579. Distinct from the other isoforms which derive from specific splicing variants, Foxp1A and Foxp1C are translated from a common mRNA, with Foxp1C being translated from an alternative initiation at methionine 251 of Foxp1A (http://www.uniprot.org/). In murine embryonic stem cells (ES), an ES-specific isoform termed Foxp1-ES is important to promote maintenance of pluripotency, while a splicing switch towards the Foxp1A isoform induces neuronal differentiation (Gabut *et al.,* 2011). This suggests that distinct FoxP1 isoforms might have very specialized roles. In the CNS, Foxp1 is expressed by projection neurons in the mouse striatum (Tamura *et al.,* 2004). While Foxp1 interacts with Foxp2 as they are co-expressed in neural structures relevant to speech and language disorders, Foxp1 is not considered a major cause of DVD (Vernes *et al.,* 2009). However, Foxp1 and Foxp2 are expressed by different populations of neurons in the mouse

cerebellar cortex (Hisaoka *et al.,* 2010). Mutations Foxp1 are associated with autism, language impairment and intellectual disability (Hamdan *et al.,* 2010; Le Fevre *et al.,* 2013; Lozano *et al.,* 2015; Palumbo *et al.,* 2013; Sollis *et al.,* 2016). Consistently, increased levels of FOXP1 mRNA expression have been observed in patients affected by autism spectrum disorders (Chien *et al.,* 2013). More recently, mice bearing a Nestin-driven conditional deletion of Foxp1, which will deplete Foxp1 in progenitor cells expressing Nestin, have been shown to display autism like behavior and gross morphological aberration of the striatum. These mice also showed abnormal neuronal morphogenesis associated with reduced excitability in the region CA1 of the hippocampus (Bacon *et al.,* 2015). To shed light on Foxp1 role in autism, Araujo and colleagues showed that Foxp1 regulates a network of autism-associated genes, therefore regulating excitability of striatal medium spiny neurons. They were also able to demonstrate that Foxp1 is required for proper ultrasonic vocalization in mice, as $Foxp1^{+/-}$ mice exhibit a reduced number of vocalization bouts and a reduction in the number and the frequency of ultrasound vocalization (Araujo *et al.,* 2015). Consistently, Precious *et al*. showed that Foxp1 is required for differentiation of medium spiny neurons during murine embryonic development (Precious *et al.,* 2016). Foxp1 is also expressed by motor neurons, where it coordinates migration in the spinal cord together with the LIM homeodomain protein Ihx1 (Palmesino *et al.,* 2010). Finally, Foxp1 has been shown to regulate cortical radial neuron migration to the cortical plate and neuronal morphogenesis in the developing cerebral cortex (Li *et al.,* 2015). In conclusion Fox factors play a prominent role in the CNS by regulating diverse aspects of neurogenesis a both during development and in the adult niches.

Sox factors and neurogenesis

The Sry-related high-mobility box (Sox) group family of 20 transcription factors subdivided into 8 groups (A-H) (Dy *et al.,* 2008). Sox proteins contain a DNA-binding domain called high-mobility group (HMG)-box which presents a high similarity with the Sry-box of sex-determining region Y *Sry*, a gene first identified for its cryical role as the sex-determining region on the Y chromosome (Koopman *et al.,* 1991). The Sox factors present specific expression patterns and have been described to play fundamental rose in lineage specification and cell fate in different compartments such as embryonic stem cells, chondrocytes, immune cells, cardiovascular system, nervous system and cancer (Abdelalim *et al.,* 2014; Kuwahara *et al.,* 2012; Lefebvre and Bhattaram, 2016; Lilly *et al.,* 2017; Vervoort *et al.,* 2013a; Vervoort *et al.,* 2013b; Weider and Wegner, 2017). Within the Sox family there are 20 members, classified into distinct groups (SoxA-SoxH) based on the degree of amino acid identity within the HMG-box. DNA-binding occurs through the three α-helices of the HMG-box domain interacting with the minor groove of the DNA (Reiprich and Wegner, 2015). All Sox proteins recognize the canonical DNA consensus sequence 5'-(A/T)(A/T)CAA(A/T)G-3, where the exact base composition will determine whether the sequence is bound preferentially by the different family members (Reiprich and Wegner, 2015). The Sox factors have been extensively characterized with regards to their role in neural development (summarized in **Table 2**).

Table 2: Sox factors involved in neurogenesis.

	Expression	Role	Reference
Sox2	Embryonic stem cells	Essential for the formation of the neuroectoderm. Sox2 binds lineage-specific genes that will be bound and activated by Sox3 in neural precursors.	(Zhao *et al.,* 2004)
	Embryonic and adult NSCs		(Bergsland *et al.,* 2011)
		Promotes proliferation, self-renewal and survival and neurogenesis	(Ferri *et al.,* 2004)
		of embryonic and adult NSC by inducing Shh, nestin and survivin	(Thiel, 2013)
Sox9	neural progenitor cells	Essential for the neurosphere formation capacity *in vitro*. Sox9	(Scott *et al.,* 2010)
	adult SVZ	inhibition by miR-124 promotes neurogenesis	(Cheng *et al.,* 2009)
Sox5	neural progenitors	Overexpression forces cells to exit of the cell cycle resulting in apoptosis by inducing Axin2	(Martinez-Morales *et al.,* 2010)
Sox21	neural progenitor cells	promotion of adult neurogenesis in *Xenopus* by inhibiting the activity of the transcription factor Hes5	(Matsuda *et al.,* 2012)

SoxC factors

SoxC factors include the transcription factors Sox4, Sox11 and Sox12. These three factors share a high level of identity within the HMG domain and the C-terminal transactivation domain (TAD) (Dy *et al.,* 2008; Penzo-Mendez, 2010) (**Figure 4**). Sox12 (known as SOX22 in human) is expressed in neural tissue and mesenchyme of human embryos, and in heart, liver spleen,

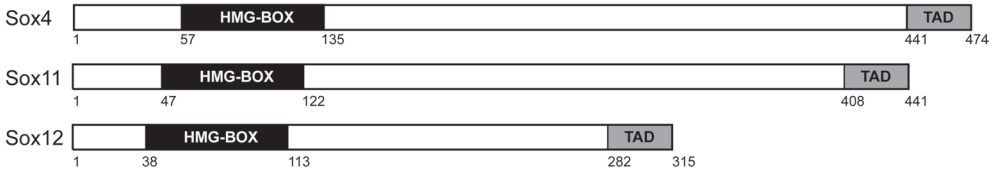

Figure 4: Schematic representation of SoxC transcription factors.

Depiction of the domains of the three members of the SoxC factors. HMG-BOX: high-mobility group (HMG)-box; TAD: transactivation domain.

pancreas and kidneys in adults (Jay *et al.,* 1997). However, Sox12 function in the neural tissue has not been characterized. Sox11 is widely expressed during embryogenesis in mouse and fetal development in human, including lung, gastrointestinal tract, kidneys spleen, central and peripheral nervous system (Dy *et al.,* 2008). Sox4 is expressed overlapping with the other SoxC factors in several tissues during embryonic development, such as mesenchymal tissues and central and peripheral nervous system (Vervoort *et al.,* 2013b). Moreover, Sox4-null mice are lethal at E14 resulting from circulatory failure due to malformation to the cardiac outflow tract (Schilham *et al.,* 1996). Additionally, Sox4 hypomorphic mice display accelerated aging and reduced cancer incidence (Foronda *et al.,* 2014). Sox4 has been shown to promote T and B cell differentiation, endocrine islet formation, osteoblast development and breast cancer (Nissen-Meyer *et al.,* 2007; Schilham *et al.,* 1997; Vervoort *et al.,* 2013a; Wilson *et al.,* 2005).

Several studies show the importance of Sox11 and Sox4, both together and individually, in regulating neurogenesis. Bergsland *et al.* demonstrated that during chicken embryonic development Sox4 and Sox11 activate the expression of pan-neuronal genes such as βIII-tubulin and MAP2, indicating how Sox4/11 function during the terminal steps of neurogenesis. In particular, Sox4 and Sox11 have been shown to directly activate the transcription of βIII-tubulin by binding to its promoter (Bergsland *et al.,* 2006). Additionally, the same study shows how Sox4 and Sox11 are transcriptionally activated by the pro-neural transcription factor Ngn2 and repressed by Id2, a passive repressor of proneural protein activity. Finally, Sox4 and Sox11 are shown to be repressed the zinc finger repressor protein REST (RE1 silencing transcription factor), which is expressed in the VZ and directly represses pro-neuronal genes (Bergsland *et al.,* 2006). Moreover, Sox4 and Sox11 have been described as essential for neural progenitor proliferation and survival as specific inactivation of SoxC genes leads to apoptosis. This is in part due to SoxC-mediated activation of Tead2 expression, a transcription factor associated with the transcriptional activators YAP and TAZ downstream the Hippo pathway (Bhattaram *et al.,* 2010). The Hippo pathway is known to restrict neuroblast proliferative potential and neuronal cell number in order to regulate brain size and to ensure timely entry and exit from neurogenesis (Poon *et al.,* 2016). In line with these findings, Mu and colleagues have shown Sox4 and Sox11 co-expression in the adult hippocampus, promoting neurogenesis from neural precursor cells. In this context, Sox4 and Sox11 directly activate the expression of the neuron-specific doublecortin (DCX) by binding to its promoter. Finally these SoxC factors have been shown to enhance the direct conversion from astrocyte to neurons induced by Ngn2 (Mu *et al.,* 2012). Sox4 and Sox11, although highly homologous, also play *non*-redundant roles by acting in discrete populations of neurons in the developing cortex: Sox11 interacts with Neurogenin1 in early born neurons thereby activating the pro-neural gene NeuroD1; while, Sox4 is expressed by intermediate progenitor cells and is required for their maintenance by interacting with Neurgenin2 to activate Tbrain2

(Chen *et al.,* 2015). Taken together, these findings help to explain how Sox4 and Sox11 promote neurogenesis by regulating the biology of neural precursor cells.

Sox4 and Sox11 are also expressed by oligodendrocyte precursors, and Sox11 is downregulated during terminal differentiation of oligodendrocytes where it promotes transcription with the transcription factor Brn-1 by binding to adjacent DNA sequences (Kuhlbrodt *et al.,* 1998). Sox4 alone has also been reported to regulate gliogenesis. In fact, prolonged expression of a *SOX4* transgene under the control of the human GFAP promoter, which drives transgene expression in radial glia and astrocytes, induces defects in the architecture of the cerebellum with consequent ataxia in mouse (Hoser *et al.,* 2007). Potzner *et al.* have shown that prolonged expression of a *SOX4* transgene under the control of the myelin basic protein (MBP) promoter, which induces Sox4 expression in differentiating oligodendrocytes, inhibits myelination in the whole CNS (Potzner *et al.,* 2007). Accordingly, Bartesaghi and colleagues demonstrated that ectopic expression of Sox4 in Schwann cells, using the promoter of the myelin protein zero (MPZ) gene and the enhancer of MBP, affects myelination and exacerbates the neuropathic phenotype in a mouse model of Charcot–Marie–Tooth 4C disease (Bartesaghi *et al.,* 2015).

Taken together these observations indicate an important role of SoxC protein family in redundantly promoting neurogenesis, and a unique role of Sox4 in regulating myelination and oligodendrocyte development.

Transcriptional regulation is a pivotal factor in regulating neurogenesis and nervous system development. Studying the role of transcription factors in NSCs increases our understanding on how NSC are finely regulated in a spatial and temporal manner.

Hypoxic-ischemic brain damage in neonates

Understanding the molecular mechanisms that regulate NSC and their response to damage is the key to develop cellular-based therapies that aim to treat insults to the CNS. Perinatal hypoxic-ischemic (HI) brain injury is a condition of impaired blood gas exchange between mother and child which causes progressive hypoxemia, hypercapnia and hypoglycemia (Bax and Nelson, 1993). HI-induced encephalopathy is the major cause of neurological disability in the newborn. HI affects 1 to 6 neonates per 1000 and represents one of the largest causes of neonatal mortality. The survivors face long-term neuronal deficits as seizures, motor deficits like cerebral palsy, sensory impairments, and mental retardation (Armstrong-Wells *et al.,* 2010; de Haan *et al.,* 2006; van Handel *et al.,* 2007). Prematurely born babies are more sensitive to perinatal insults due to an underdevelopment of several organ systems which makes them more sensitive to oxygen imbalances and inflammation (Kehrer *et al.,* 2005). The brains of most term neonates are affected in gray matter areas, while preterm babies suffer mostly from

white matter damage. The latter occurs as a result of oligodendrocyte precursor cells being more sensitive to insults than mature oligodendrocytes (Back *et al.,* 2002; Haynes *et al.,* 2003). HI may occur during pregnancy, intra-partum or after delivery (Graham *et al.,* 2008; McLean and Ferriero, 2004; Volpe, 2001). Risk-factors for HI include prepartum conditions such as diabetes, placental insufficiency and pregnancy-induced hypertension, intrapartum events like placental abruption, compression of the umbilical cord and uterine rupture and post-partum events like respiratory deficiency or cardiac failure (Badawi *et al.,* 1998a, b; Milsom *et al.,* 2002).

Depending on the length and the cause of HI, events like anaerobic metabolism and ischemia will take place. Due to the reduced blood flow, levels of glucose and glycogen are depleted, followed by a drop in adenosine triphosphate (ATP). The reduced ATP levels results in failure of the Na^{2+}/K^{+} pump which causes cell-depolarization, with subsequent massive efflux of glutamate. This causes excitotoxicity, a major cause of neuronal and oligodendrocyte death. Neuronal depolarization and activation of several glutamate receptors (including the NMDA channel) induces Ca^{2+} influx subsequently activating several intracellular enzymes such as phospholipases, proteases, DNA nucleases resulting in membrane, protein and DNA degradation respectively, with subsequent formation of free radicals like nitrous oxide (Blomgren *et al.,* 2001; MacDonald *et al.,* 2006; Northington *et al.,* 2001; Takita et al., 2004; Vannucci *et al.,* 2001). These cascades will eventually lead to the activation of the immune system which, together with a disrupted blood-brain-barrier, leads to influx of peripheral immune cells and neuroinflammation, which exacerbates brain damage (Chew *et al.,* 2006; Dommergues *et al.,* 2000). Currently, clinically available treatments are limited to hypothermia (whole body cooling by 2- 4 °C) which has to be applied within 6 hours after onset of the insult to be (mildly) effective (Gluckman *et al.,* 2005; Jacobs *et al.,* 2007).

It has been shown previously that HI induces an increase of NSC proliferation in the murine SVZ (Bartley *et al.,* 2005; Iwai *et al.,* 2003; Yang and Levison, 2006). Felling and colleagues showed that this increased proliferation is accompanied by enhanced multipotency of SVZ-derived neurospheres and an increase in expression levels of epidermal growth factor receptor (EGFR) and Notch1 in the SVZ (Felling *et al.,* 2006). However, this boost in neurogenesis is dampened by an increase in apoptosis of NSCs in the SVZ, as indicated by activation of caspase-3, a marker of apoptotic cells (Romanko *et al.,* 2007). These regenerative events imply that the formation of new neurons could aid in replacing the lost tissue, however this response is too limited to be sufficient. Therefore treatments that aim to enhance neuroregeneration after HI should be investigated.

Cell-based therapies for neonatal hypoxia-ischemia (HI): neural stem cells, mesenchymal stem cells and extracellular-vesicle transplantation

In order to restore the function of the damaged CNS after HI, cell-based transplantation approaches have been explored in preclinical settings. These strategies either aimed at replacing the injured tissues or at stimulating the endogenous neurogenesis potential. One strategy consists in providing directly neural precursor cells to the damaged brain. Several kinds of progenitors have been explored for their potential. A rather coarse approach consists of transplanting undissociated patches of cortical tissue to rats with HI, which contain different kinds of neural progenitors. In fact, HI-injured rats have been transplanted at 1 week after the insult with fetal cortex cells deriving from E13 rats; the grafts survived and developed acetylcholinesterase-positive fibers crossing the host/transplant interface, an indication of integration of the graft into the host brain (Elsayed *et al.,* 1996). Accordingly, Jansens and colleagues showed in neonatal rats that fetal neocortical grafts transplanted at 3 days post-HI express nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), choline acetyl transferase (ChAT), cholecystokinin (CCK), and glial fibrillary acidic protein (GFAP), indicating extensive host-graft integration. Additionally, the recipient animals presented reduced sensorimotor impairment associated with the brain injury (Jansen *et al.,* 1997). A more refined approach is represented by the transplantation of a defined population of isolated precursors. Titomanlio *et al*. transplanted murine neurosphere-derived precursors into a murine model of excitotoxicity-induced brain injury. The animals were treated early (4 h) or 3 days after injury. The transplanted precursor cells grafted into the damaged brains, migrated towards the lesion site, and differentiated towards $NG2^{+}$-oligodendrocytes and $MAP2^{+}$-neurons at 42 days after treatment. Even though the cells underwent apoptosis a few weeks later, this treatment resulted in a reduction of lesion size and improvement of memory performance after HI (Titomanlio *et al.,* 2011). The hypothesis that transplanted NSCs integrate into the host brain is supported by Park and colleagues, who showed how murine NSCs seeded onto a polymer scaffold were transplanted into the HI infarction cavities and resulted in interaction between NSCs and the damaged murine brain tissue. The transplanted NSCs differentiated towards neurons and formed long-distance neuronal connections from the scaffold towards regions of the contralateral hemisphere (Park et al., 2002). The use of xenograft *human* NSCs in HI-injured rats has been explored by Daadi and colleagues. The transplanted NSCs differentiated towards neurons, astrocytes and oligodendrocytes, and enhanced axonal sprouting. Importantly, the NSC-treated animals also improved their sensorimotor performance (Daadi *et al.,* 2010).

Another cell type that has been investigated for its therapeutic potential is the mesenchymal stromal cell (MSC). These cells can be isolated from different tissues, including the bone marrow, umbilical cord and adipose tissue (Salem and Thiemermann, 2010). MSCs are capable of differentiating towards adipocytes, chondrocytes and osteoblasts. Moreover they are known for secreting a variety of soluble factors ranging from neurotrophic factors

(VEGF, FGF2, BDNF and NGF) to immunomodulatory (IL-10, TGFβ and IFNγ) (Crigler *et al.,* 2006; Deans and Moseley, 2000; Salem and Thiemermann, 2010; Zhang *et al.,* 2013). Additionally, MSCs are considered not to be immunogenic. Several clinical trials are now investigating the possible use of MSCs in treating the most diverse conditions, spanning from arthritis to graft-versus-host disease (www.clinicaltrials.gov). Intracranial transplantation of MSCs has been shown beneficial for the sensorimotor function of HI-injured animals (van Velthoven *et al.,* 2010a, c). Moreover, MSC transplantation induces proliferation in area of the hippocampus, accompanied by formation of new neurons and oligodendrocytes. MSCs induce remodeling of the corticospinal tract and reduce the lesion area (van Velthoven *et al.,* 2010a, c). Interestingly, the transplanted MSCs change their expression of secreted proteins like IL10, IL1β or NGF after exposure to HI brain extracts, indicating that MSCs are capable of responding to the damaged brain milieu (van Velthoven *et al.,* 2011). Supporting the beneficial effect of intracranial transplanted MSCs, MSCs that are administrated through the nasal route are able to migrate to the lesion site within 2 hours from administration, probably by migrating towards chemoattractants generated within the HI lesion (van Velthoven *et al.,* 2010b). Distinct from NSCs, MSCs do not integrate into the host brain after HI, but rather induce endogenous precursor proliferation and differentiation. In fact, labeled MSCs do not express neuronal or glial markers, but induce the number of DCX-positive neuroblasts into the SVZ and reduce neuroinflammation at the damage site (Donega *et al.,* 2014). This raises the hypothesis that MSCs might exert their role through their secretome. Exosomes, small spherical vesicles (around 50 nm in diameter) limited by a lipid bilayer, contain a range of numerous proteins and RNA molecules (Mittelbrunn and Sanchez-Madrid, 2012; Valadi et al., 2007). These vesicles bud from the cell membrane of virtually any type of eukaryotic cell, and after being secreted in the extracellular space fuse with the membrane of the target cell, releasing their biologically active content (Maas *et al.,* 2017). A few studies have suggested that MSC-derived exosomes have the potential to be utilized as a novel cell-free therapy for brain damage. The advantage of using this cell-free approach would overcome the limitations of using living cells to treat HI. For example, the use of cells carries an inherent risk of tumorigenesis, as the transplanted cells could potentially give origin to malignancies. Moreover, the transplantation of heterologous cells might elicit an immune response with consequent rejection of the graft. To this end, the use of exosomes might overcome this limitations. Moreover, these vesicles could be isolated and stocked in large quantities, diminishing the wait for treatment (whereas cells would first need to be cultured to reach the desired quantity for transplantation) Xin *et al.* have shown that MSC-derived exosomes induce neurite outgrowth of endogenous neural cells through these vesicles and thereby promote functional recovery and neural plasticity in a rat model of stroke (Xin *et al.,* 2013a; Xin *et al.,* 2013b). Finally, MSC-derived extracellular vesicles, which comprise also exosomes, have been demonstrated to protect the fetal brain in an ovine model of HI. In fact, these vesicles reduced seizure burden, pressure sensitivity and white matter injury

(Ophelders *et al.,* 2016). Taken together, these observations indicate that exosomes might be developed as a cell-free tool to treat HI brain damage.

Aim of the thesis

Understanding molecular regulation of NSC differentiation is the key to develop novel treatments for conditions affecting the CNS such as HI. To this end this thesis describes the role of two transcription factors, Foxp1 and Sox4, in regulating NSC differentiation. Moreover, it describes three different strategies to regenerate the neonatal brain after HI: NSC and MSC transplantation and exosome administration. In **Chapter 2,** Foxp1 is described as a pivotal transcription factor in promoting NSC differentiation *in vitro* and *in vivo* by regulating neurogenesis-specific genes and by repressing the Notch ligand Jagged1. The development of treatments for HI can range in different directions, from the transplantation of neural progenitors or mesenchymal stem cells to the use of extracellular vesicles. In the second part of this thesis we explore the potential of this tools for HI treatment. **Chapter 3,** describes the role of Sox4 in NSC differentiation, specifically how this transcription factor inhibits NSC differentiation *in vitro* towards oligodendrocytes by directly activating the expression of Hes5, a transcription factor downstream of the Notch pathway. In **Chapter 4**, NSCs are shown as a tool to reduce sensorimotor impairment after HI by differentiating towards neurons in the lesion area and by dampening neuroinflammation through their secretome. In **Chapter 5**, MSCs modified to express an array of pro-repair secreted genes are tested for their capacity to induce NSC differentiation *in vitro* and transplanted in neonatal HI mice to investigate their regenerative potential. **Chapter 6** evaluates the hypothesis that exosomes can be utilized as a tool to treat CNS injuries. In **Chapter 7** the findings described in this thesis are discussed, speculating on the role of Foxp1 and Sox4 in regulating NSC biology. Moreover, the different approaches to treat the brain after HI analyzed here are put in perspective within the current field. Taken together, the findings described in this thesis shed light on novel aspects of NSC regulation and describe new tools to regenerate brain damage after HI.

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CHAPTER 2

Foxp1 promotes embryonic neural stem cell differentiation by repressing Jagged1 expression.

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Abstract

Foxp1 has been demonstrated to promote neuronal migration and morphogenesis, as well as neurogenesis in mice. Mutations in Foxp1 have been linked to neurodevelopmental disorders including intellectual disability and autism, however the underlying molecular mechanisms remain ill-defined. Here we demonstrate that Foxp1 is expressed in embryonic neural stem cells (NSCs) and is upregulated during differentiation *in vitro*. Modulation of Foxp1 expression in NSCs impacts both neuron and astrocyte differentiation *in vitro*. Transplantation of Foxp1-knockdown NSCs in neonatal mice after hypoxia-ischemia (HI) challenge demonstrated that Foxp1 is required for neuronal differentiation *in vivo*. Foxp1-knockdown prevented NSC-mediated improvement of motor function after HI. Additionally, Foxp1-knockdown *in utero* reduced NSC differentiation during corticogenesis. RNA- and chromatin immunoprecipitation (ChIP)-sequencing showed that Foxp1 regulates neurogenesis genes and represses the Notch pathway. Expression of the Notch-ligand Jagged1 was repressed by Foxp1, while blockade of Jagged1 in Foxp1-knockdown cells rescued neuronal differentiation of NSCs *in vitro*. Together these data support a novel role for Foxp1 in regulating embryonic NSC differentiation by modulating Notch signaling.

Introduction

Neural stem cells (NSCs) are mutipotent progenitor cells found in the developing and adult brain in specialized niches of the subgranular zone of the hippocampal dentate gyrus and the subventricular zone (SVZ). During cortical development the radial glial cells are considered embryonic NSCs acting as neural progenitor/stem cells at the ventricular zone (VZ) and, while migrating towards the cortical plate (CP), differentiate giving origin to neurons, astrocytes and oligodendrocytes (Fishell and Kriegstein, 2003; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). NSCs represent a promising candidate for transplantation in the treatment of pathophysiological conditions of the central nervous system (CNS) such as Alzheimer, multiple sclerosis, cerebral stroke or hypoxic-ischemic encephalopathy (Bacigaluppi *et al.,* 2016; Blurton-Jones *et al.,* 2009; Braccioli *et al.,* 2016; Daadi *et al.,* 2010; Martino and Pluchino, 2006; Park *et al.,* 2002). Regulation of NSC differentiation includes involvement of the Notch signaling pathway that has been widely described as a key factor promoting neural progenitor maintenance and modulation of NSC fate decision in both embryonic and adult NSCs (Ables *et al.,* 2011; Louvi and Artavanis-Tsakonas, 2006). Forkhead box p1 (Foxp1) is a transcription factor belonging to the forkhead family of transcription factors and has been implicated in the development of the heart, lung, esophagus and immune system, as well as in cancer (Bacon and Rappold, 2012). During embryonic neural development, Foxp1 has been demonstrated to promote neuronal migration and morphogenesis, as well as differentiation of medium spiny neurons (Li *et al.,* 2015; Precious *et al.,* 2016). Mutations in Foxp1 have been linked to neurodevelopmental disorders including speech defects, intellectual disability and autism (Hamdan *et al.,* 2010; Horn *et al.,* 2010; Le Fevre *et al.,* 2013; Lozano *et al.,* 2015; Palumbo *et al.,* 2013; Sollis *et al.,* 2016). Additionally, Foxp1 has recently been shown to regulate a network of autism-associated genes in the hippocampus and striatum, and heterozygous $Foxp1^{+/-}$ mice exhibit vocal communication defects. However, the role of Foxp1 in NSCs has not been addressed (Araujo *et al.,* 2015). A recent study from Bacon and colleagues showed that recombinase Cre-mediated deletion of Foxp1 in Nestin-positive cells causes autism-like behavior and gross morphological defects in the striatum, detected from early postnatal age onwards (Bacon *et al.,* 2015).. However, the question as to whether Foxp1 regulates embryonic neural progenitor differentiation remains open. In this study we have investigated the role of Foxp1 in regulating embryonic NSC differentiation. Through both *in vitro* and *in vivo* analyzes combined with global transcriptional profiling, here we identify Foxp1 as a driver of NSC differentiation towards astrocytes and neurons. Furthermore, we define Foxp1-mediated repression of *Jagged1*, a key ligand of the Notch pathway, as being a requirement for NSC differentiation. Taken together, these findings highlight Foxp1 as a pivotal transcription factor in regulating embryonic NSC differentiation, defining a mechanism for its role in neurogenesis.

Results

Foxp1 promotes embryonic neural stem cell differentiation towards astrocytes and neurons *in vitro*

While previous studies have implicated Foxp1 in neurogenesis and the pathogenesis of autism, speech defects and other intellectual disabilities; little is known about the molecular mechanisms underlying these effects. Here we sought to investigate the role of this transcription factor in neural stem cell (NSC) functionality. To this end we first determined whether Foxp1 expression was regulated during murine NSC differentiation. NSCs derived from the prefrontal cortex of E14.5 CD-1 embryos were differentiated *in vitro* for 10 days by growth-factor withdrawal (as described in Supplemental Experimental Procedures). Foxp1 expression levels were assessed every 2 days and a significant increase in Foxp1 mRNA was observed after 2 days of differentiation which was sustained for at least 6 days (**Figure 1A**). Consistently, Foxp1A (79kDa) and Foxp1C (50kDa) protein isoforms were detected, both showing a significant increase during differentiation of the NSCs (**Figures 1B and 1C**). This increase in Foxp1 levels during the early phase of differentiation indicates a potential functional role for Foxp1 in regulating NSC lineage choices. To evaluate this, shRNA-mediated Foxp1 knockdown (KD) was utilized to deplete NSCs of both Foxp1A and Foxp1C isoforms (**Figure 1D**). Firstly, to assess whether Foxp1 KD affects NSC proliferation, the percentage of Ki67-positive cells were analyzed in presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) that promote NSC proliferation and no differences were observed (**Figures S1A and S1B**). Secondly, to determine whether Foxp1 KD impairs differentiation, NSCs were differentiated for 5 days towards neurons, astrocytes and oligodendrocytes. Differentiation was quantified by measuring lineage-specific marker expression by immunofluorescence: βIII-tubulin, glia fibrillary acidic protein (GFAP) and neural/glial antigen 2 (NG2) respectively (Eng and Ghirnikar, 1994; Memberg and Hall, 1995; Polito and Reynolds, 2005). Foxp1 KD significantly reduced the percentage of βIII-tubulin- and GFAP-positive cells (**Figures 1E, 1G and 1H**), while the percentage of NG2-positive cells was unaffected when compared to control (**Figures S1C and S1D**). Consistently, Foxp1 KD increased the expression (per cell) of the stem cell/progenitor marker Nestin when compared to control (**Figures 1F and 1I**) (Lendahl *et al.,* 1990). This indicates that reduction in neuronal and astrocyte differentiation observed after Foxp1 KD is accompanied by maintenance of progenitor-like characteristics. Conversely, ectopic expression of Foxp1 (**Figure 1J**) increased the percentage of βIII-tubulin- and GFAP-positive cells when compared to control (**Figures 1K-1M**). Taken together, these data demonstrate that Foxp1 is required for NSC differentiation *in vitro,* specifically to both neuronal and astrocyte lineages but not towards oligodendrocytes.

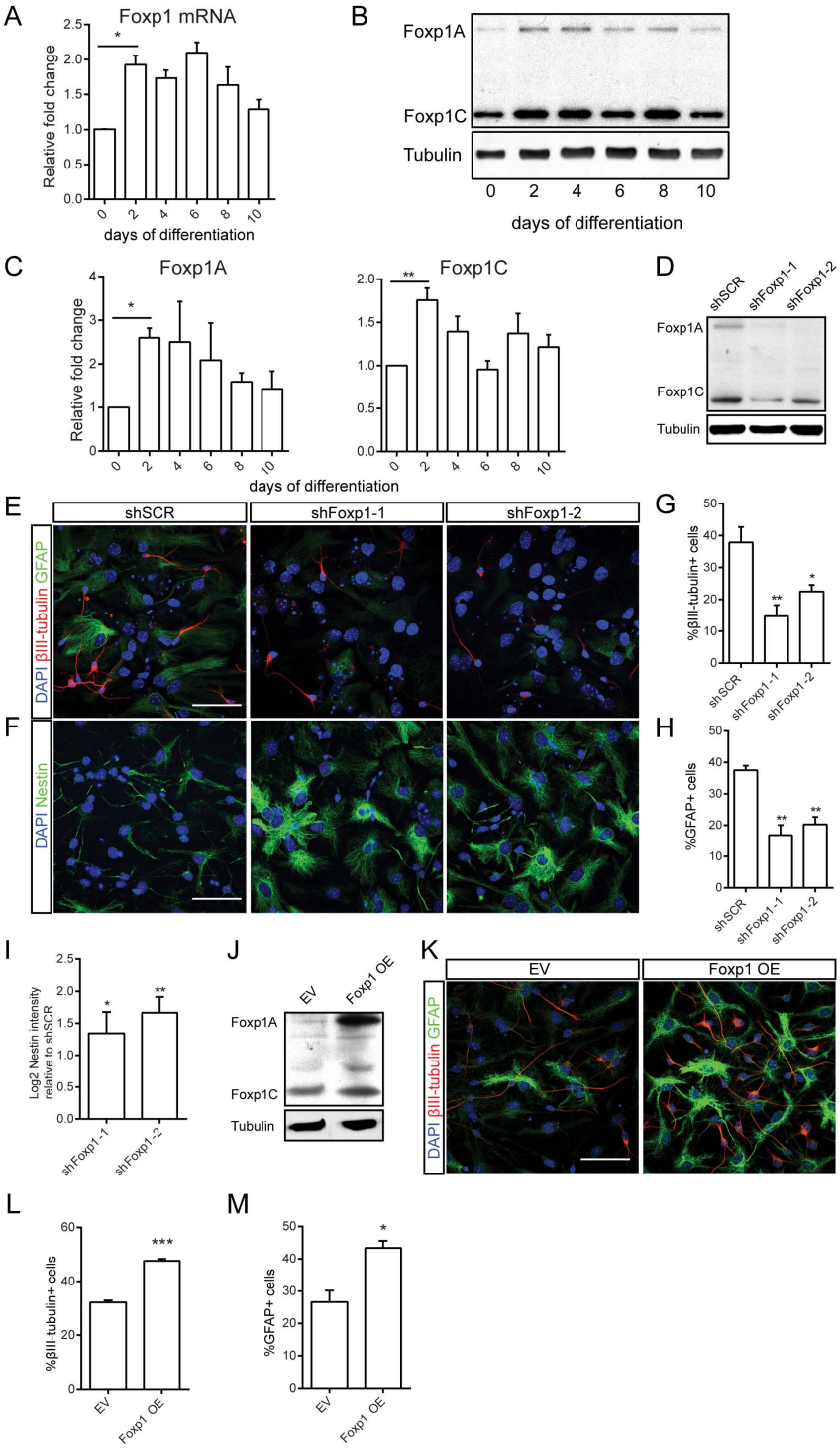
A
Foxp1 mRNA
Relative fold change
days of differentiation
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Figure 1. Foxp1 regulates NSC differentiation *in vitro*.

(A) NSCs were differentiated for 10 days and RNA and protein were collected. Measure of Foxp1 mRNA levels during differentiation (n=3) *=p<0.05. **(B)** Representative Western blot showing the expression of Foxp1A and Foxp1C isoforms. **(C)** Quantification of B (n=3). *=p<0.05, **=p<0.01. **(D)** Representative western blot showing efficient knockdown of Foxp1A and Foxp1C with 2 independent shRNAs. **(E)** Foxp1 KD NSCs were differentiated for 5 days. Representative confocal images showing the expression of the neuronal marker βIII-tubulin (red) and the astrocyte marker GFAP (green). DAPI co-stained nuclei in blue (bar=50 μm). **(F)** Confocal images showing the expression of the neural progenitor marker Nestin (green). DAPI co-stained nuclei in blue (bar=50 μm). **(G)** and **(H):** quantification of E (n=3) *=p<0.05, **=p<0.01. **(I)** quantification of F. (n=3) *=p<0.05, **=p<0.01. **(J)** Representative Western blot showing the overexpression of Foxp1. **(K)** Foxp1 overexpressing NSCs were differentiated for 5 days. Representative confocal images showing the expression of the neuronal marker βIII-tubulin (red) and the astrocyte marker GFAP (green). DAPI co-stained nuclei in blue (bar=50 μm). **(L)** and **(M)** quantification of K (n=3) *=p<0.05, ***=p<0.001. Error bars represent SEM. See also Figure S1.

Foxp1 is required for *in vivo* differentiation of NSCs in a hypoxic-ischemic brain damage model

To investigate whether Foxp1 is also required for NSC differentiation *in vivo*, Foxp1-depleted NSC genetically labeled with DsRed were transplanted in a murine model of neonatal hypoxic-ischemic (HI) brain injury. As we have previously described, this model induces unilateral damage to hippocampus, neocortex and striatum resulting in sensorimotor impairment (van der Kooij *et al.,* 2010). Nine days after birth, animals underwent right-carotid artery occlusion followed by systemic hypoxia (as described in Experimental Procedures) to induce brain injury. At 10 days after HI, Foxp1 KD NSCs were transplanted intracranially at the lesion site in the ipsilateral hippocampus, and the animals were subsequently sacrificed 5 days later to assess the fate of the transplanted NSCs (**Figure 2A**). Another group of animals survived until day 28 to assess the effect of Foxp1 KD NSC transplantation on motor behavior (**Figure 2A**).

We have previously demonstrated that transplanted NSCs in HI animals localize around the ipsilateral hippocampus and differentiate towards the neuronal lineage by expressing the neuroblast marker doublecortin (DCX) as soon as 3 days after transplantation (Braccioli *et al.,* 2016). Expression of GFAP in the transplanted cells is absent at this time, indicating no differentiation of the transplanted NSCs towards the astrocyte lineage (Braccioli *et al.,* 2016). **Figure 2B** shows that Foxp1 KD NSCs were still detectable in the hippocampal area at 5 days after transplantation, however there was a significantly reduced percentage of DCX-DsRed-positive NSCs when compared to control NSCs (shSCR NSCs) (**Figures 2B and 2C**). These data support the *in vitro* findings that Foxp1 is also required for neuronal differentiation of (transplanted) NSCs *in vivo*.

To test the functional consequences of Foxp1 KD in transplanted NSC after HI, an additional group of animals was assessed for sensorimotor function utilizing the cylinder rearing test at 28 days after HI (18 days after transplantation) (**Figure 2A**) (Schallert *et al.,* 2000; van der Kooij *et al.,* 2010). We have previously shown that NSC transplantation ameliorates HI-induced motor impairment under these experimental conditions (Braccioli *et al.,* 2016).

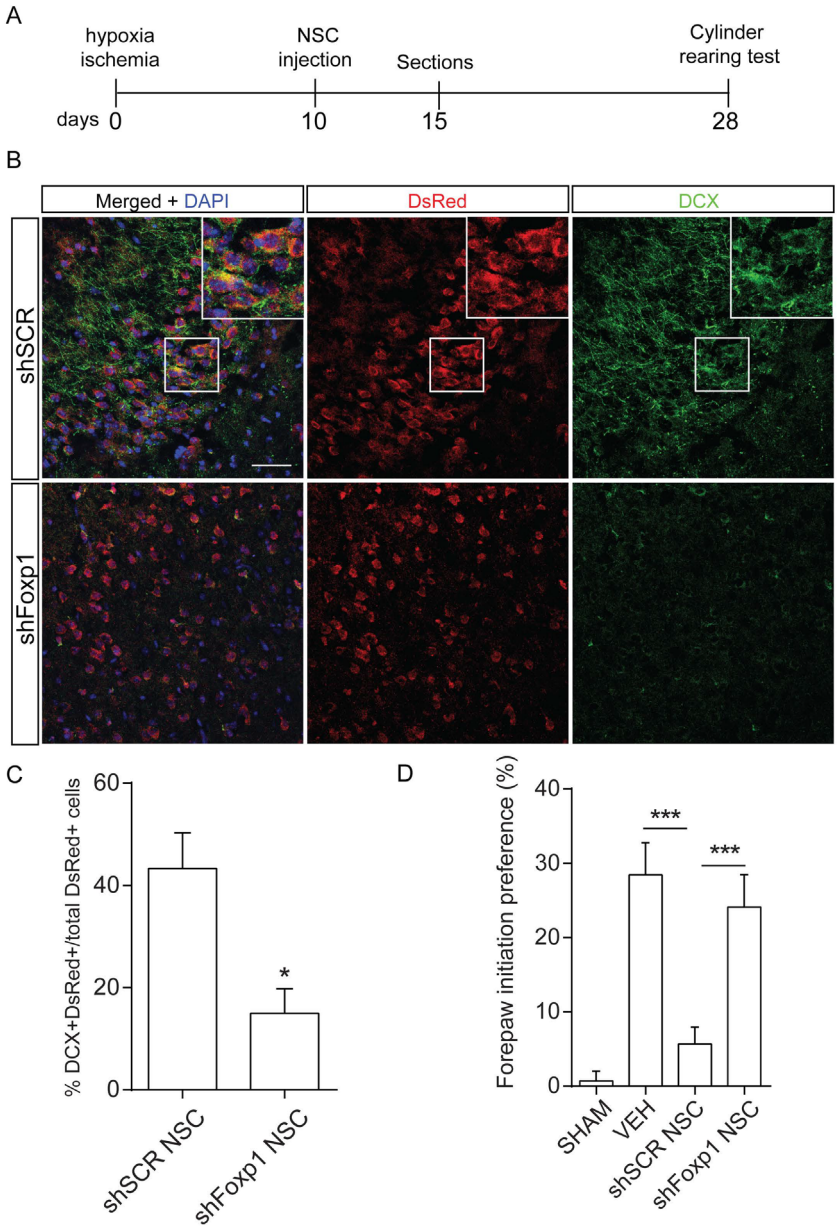


Figure 2. Foxp1 is required for NSC differentiation *in vivo* after transplantation into HI mice.

(A) Experimental set-up showing the day of sham or HI operation (day 0, postnatal day 9), intracranial NSC treatment (day 10) and the day of termination for histological sections (day 15). In another group of animals cylinder rearing test was performed at day 28. **(B)** Representative Z-stack confocal images of the hippocampus showing the presence of the transplanted DsRed NSCs (red) and the expression of the neuroblast marker DCX (green). Yellow indicates colocalization. DAPI co-stained nuclei in blue (bar=50 µm). Insets show magnification of the boxed area. **(C)** Quantification of double-positive cells in (B) (mice per group: shSCR NSC n=3. shFoxp1 NSC n=3). *= p<0.05. **(D)** Results of the cylinder rearing test at 28 days showing that Foxp1 is required for the improvement in motor behavior mediated by NSCs after HI (mice per group: SHAM n=9, VEH n=9, shSCR-NSC n=10, shFoxp1-NSC n=8). ***=p<0.001. Error bars represent SEM.

The control HI animals treated with vehicle (VEH) showed impaired sensorimotor function (i.e. increased preference for the non-impaired forepaw) when compared to sham-control animals (SHAM). Animals treated with control (shSCR) NSCs displayed a potent improved performance when compared to VEH-treated HI animals which is in line with our previous findings (Braccioli *et al.,* 2016). Consistent with the histological findings in Figure 2b-c, Foxp1 KD completely reduced the improvement in motor behavior mediated by NSC transplantation after HI when compared to shSCR control NSCs (**Figure 2D**). Taken together, these data indicate that Foxp1 promotes differentiation of transplanted NSCs towards the neuronal lineage *in vivo,* and Foxp1 is also required for the functional improvement of the animals mediated by NSC transplantation after HI.

Foxp1 is required for NSC migration and differentiation during cortical development

During embryonic development of the cortex, NSCs residing in the ventricular zone (VZ) differentiate into neurons migrating to the more superficial cortical plate (CP) where they establish functional connections (Kriegstein and Alvarez-Buylla, 2009). To assess whether Foxp1 is expressed by NSCs in the VZ, cortices from day 12 (E12) and 14 (E14) embryos were stained both for the neural precursor/neural stem cell marker Sox2 (Ellis P *et al.,* 2004) and Foxp1. As expected, at both E12 and E14 Sox2 expression was mainly localized at VZ (**Figure 3A**). Interestingly, Foxp1/Sox2-positive cells were found at the VZ of both E12 and E14 cortices (**Figure 3A**). Additionally, Foxp1 was found to be expressed in the CP, consistent with previous reports describing a role for Foxp1 in differentiated neurons (Araujo *et al.,* 2015; Precious *et al.,* 2016). To evaluate whether Foxp1 KD affects NSC differentiation during cortical development, E14.5 embryos were electroporated *in utero* with shRNA vectors together with GFP in order to transduce the NSCs present at the VZ, followed by immunohistochemical analysis of neurons derived from these progenitors. At E17.5 the majority of GFP-positive cells were found at the CP. In contrast, Foxp1 KD showed an increase of GFP-positive cells at the intermediate zone (IZ), and a reduction of GFP-positive cells at the CP/marginal zone (MZ) when compared to control (**Figures 3B and 3C**). These data indicate that Foxp1 KD reduces migration of the differentiating neurons deriving from the VZ, as reported previously (Li *et al.,* 2015). To assess whether this phenotype was accompanied by reduced differentiation, we analyzed the expression of Ctip2, a transcription factor expressed by a subtype of post-mitotic cortical neurons during development (Leone *et al.,* 2008). Upon Foxp1 KD, a reduction of the percentage of $Ctip2^{+}GFP^{+}$-cells was observed compared to shSCR control (**Figures 3D and 3E**), indicating that Foxp1 is required for the differentiation of NSCs towards post-mitotic cortical neurons. Taken together, these observations indicate that Foxp1 is required for differentiation of NSCs during corticogenesis.

Genome-wide analysis of Foxp1 DNA-binding in NSCs

While our data support a functional role for Foxp1 in driving neurogenesis, the molecular mechanisms underlying this remain obscure. To gain more insight, Foxp1 chromatin

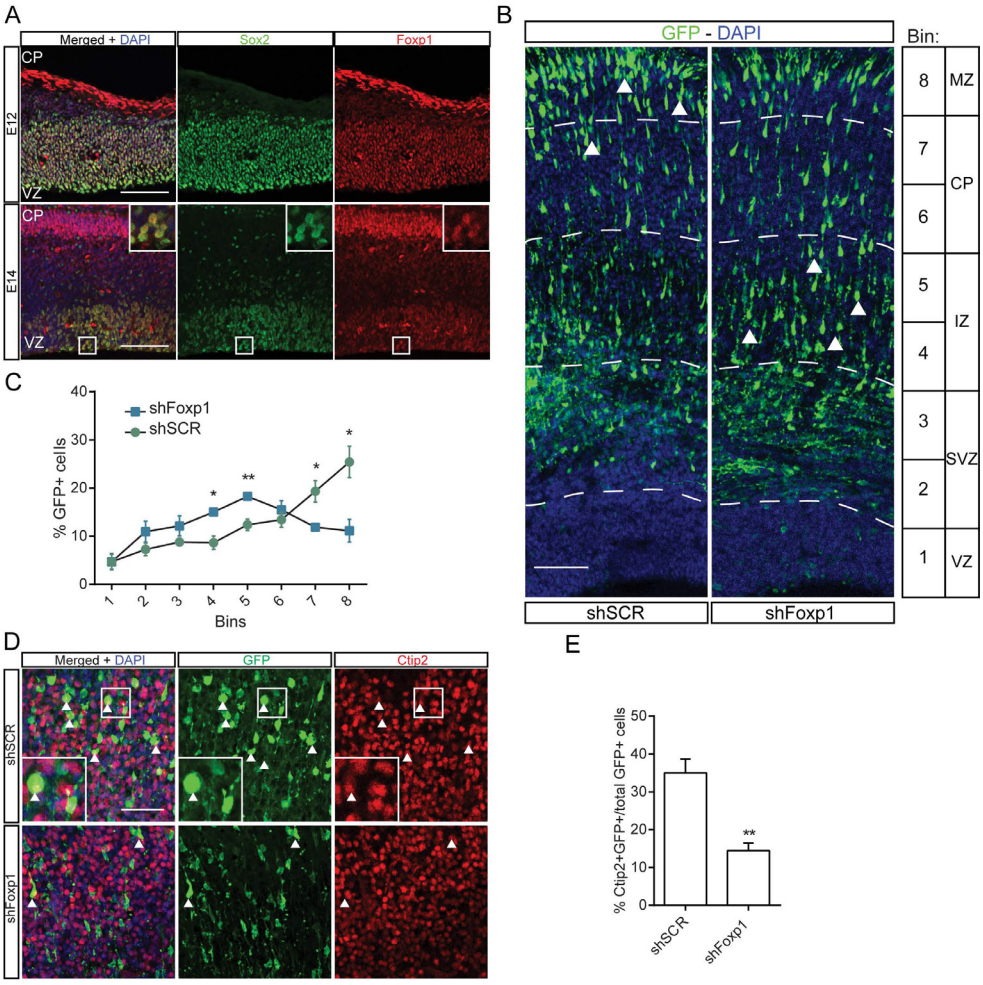


Figure 3. Foxp1 is required for radial glia development during embryogenesis.

(A) E12 and E14 motor cortices were stained for Foxp1 and Sox2 to assess the expression of Foxp1 by NSCs during development. Z-stack confocal images showing co-expression of Foxp1 (red) and Sox2 (green) at the VZ both at E12 and E14. Expression of Foxp1 can be detected also at the CP. Inserts show magnification of the boxed area. DAPI co-stained nuclei in blue (bar=100 µm). **(B)** E14.5 cortices were electroporated *in utero* with shRNAs against Foxp1 in combination with GFP. Animals were terminated at E17.5. Representative Z-stack confocal images of the motor cortex showing GFP^+ neurons (green), originating from the VZ. Foxp1 KD induces accumulation of neurons in the IZ and reduction of neurons in the cortical plate (CP)/marginal zone (MZ) (white arrowheads). SVZ: subventricular zone. IZ: intermediate zone. The cortex has been subdivided in 8 bins for quantification. DAPI co-stained nuclei in blue (bar=25 µm). **(C)** Quantification of B (mice per group: shSCR n=3, shFoxp1 n=3). *=p<0.05, **=p<0.01. **(D)** Representative Z-stack confocal images showing $Ctip2^+GFP^+$ cells (white arrowheads) in the cortex upon Foxp1 depletion. Insets show magnification of the boxed area. Green: Ctip2. Red: GFP. DAPI co-stained nuclei in blue (bar=50 µm). **(E)** Quantification of D (mice per group: shSCR n=3, shFoxp1 n=3). **=p<0.01. Error bars represent SEM.

immunoprecipitation from NSCs followed by high-throughput sequencing (ChIP-seq) was performed. Foxp1-bound loci were successfully identified as exemplified by peaks mapped at the region of the *Bik* gene, which we previously characterized as a Foxp1 target in human colon carcinoma cells (van Boxtel *et al.,* 2013) (**Figure 4A**). Analysis of genomic distribution of Foxp1-binding sites showed that binding events are enriched at promoter regions, 5'-untranslated regions (5'-UTR) and transcription start sites (TSS) when compared to random genomic regions (**Figure 4B**). Additionally, binding events were found centrally enriched around the TSS (**Figure 4C**). *De novo* motif discovery was performed and identified Forkhead motif, in addition to other co-occurring motifs including Sox3, Nr2e1 and Rfx2 (**Figure 4E**). The Foxp1/Forkhead-consensus DNA binding motif was found centrally enriched within peaks, further confirming sequence-specific binding (**Figure 4D**).

To identify which genes are associated with Foxp1-bound regions, the genes which TSS was within 1 kilobase (kb) from Forkhead-motif centered peaks were selected for gene-ontology (GO) analysis. Foxp1-bound genes showed a significant association with abnormalities in the central nervous system, abnormalities of higher mental function and cognitive impairment. This is in line with previous studies supporting association of Foxp1 mutations with autism, intellectual disability and speech defects (Horn *et al.,* 2010). Taken together, these data show that Foxp1 associates to the promoter regions of genes involved in diseases of the CNS.

Foxp1 regulates neurogenesis and Notch signaling pathway genes

To identify genes that are transcriptionally regulated by Foxp1 we evaluated the effect of Foxp1 KD on transcript expression in NSCs by RNA-sequencing (RNA-seq). We confirmed that Foxp1 mRNA levels were reduced after Foxp1 KD (**Figure 5A**). Analysis of RNA-seq data showed that upon Foxp1 KD 472 genes were found to be significantly induced by Foxp1, and 617 genes to be repressed by Foxp1 (**Figure 5B**). To identify which genes were likely directly regulated by Foxp1 in NSCs, the set of differentially expressed genes was overlapped with the set of genes whose TSS is within 25 kb from Forkhead-motif centered peaks derived from the ChIP-seq analysis. Of the Foxp1-regulated genes, 210 were found to be both induced and bound by Foxp1, whereas 274 were found to be repressed and bound by Foxp1 (**Figures 5C and 5D**).

To gain further insight into the biological processes potentially regulated by Foxp1, GO-term analysis was performed using the subset of Foxp1-bound and transcriptionally regulated target genes. Significant association was observed with neurogenesis, regulation of synapse organization and nervous system development processes (**Figure 5E**). Interestingly, also an association with the Notch signaling pathway was found (**Figure 5E**). Since Notch signaling is important for the regulation of NSC differentiation (Ables *et al.,* 2011; Louvi and Artavanis-Tsakonas, 2006) we wished to determine whether Foxp1 may then act as a repressor or inducer of the Notch pathway in NSCs. To this end, we performed a weighted gene-set

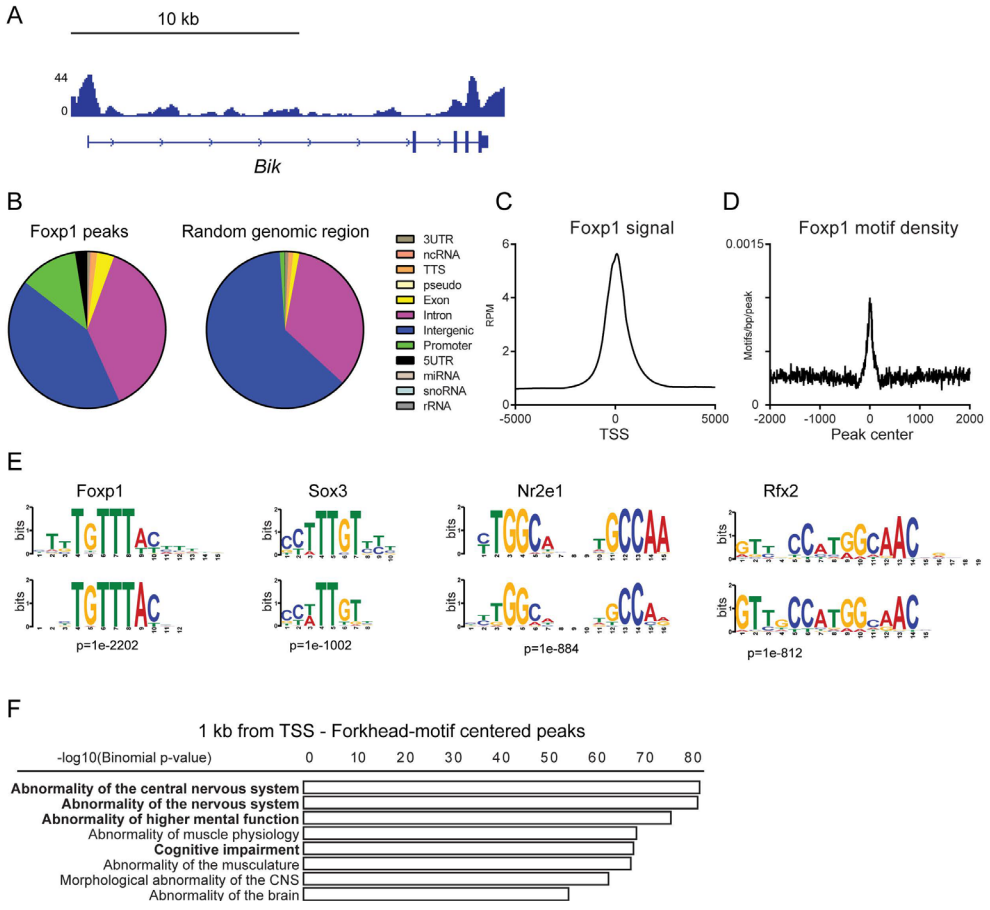
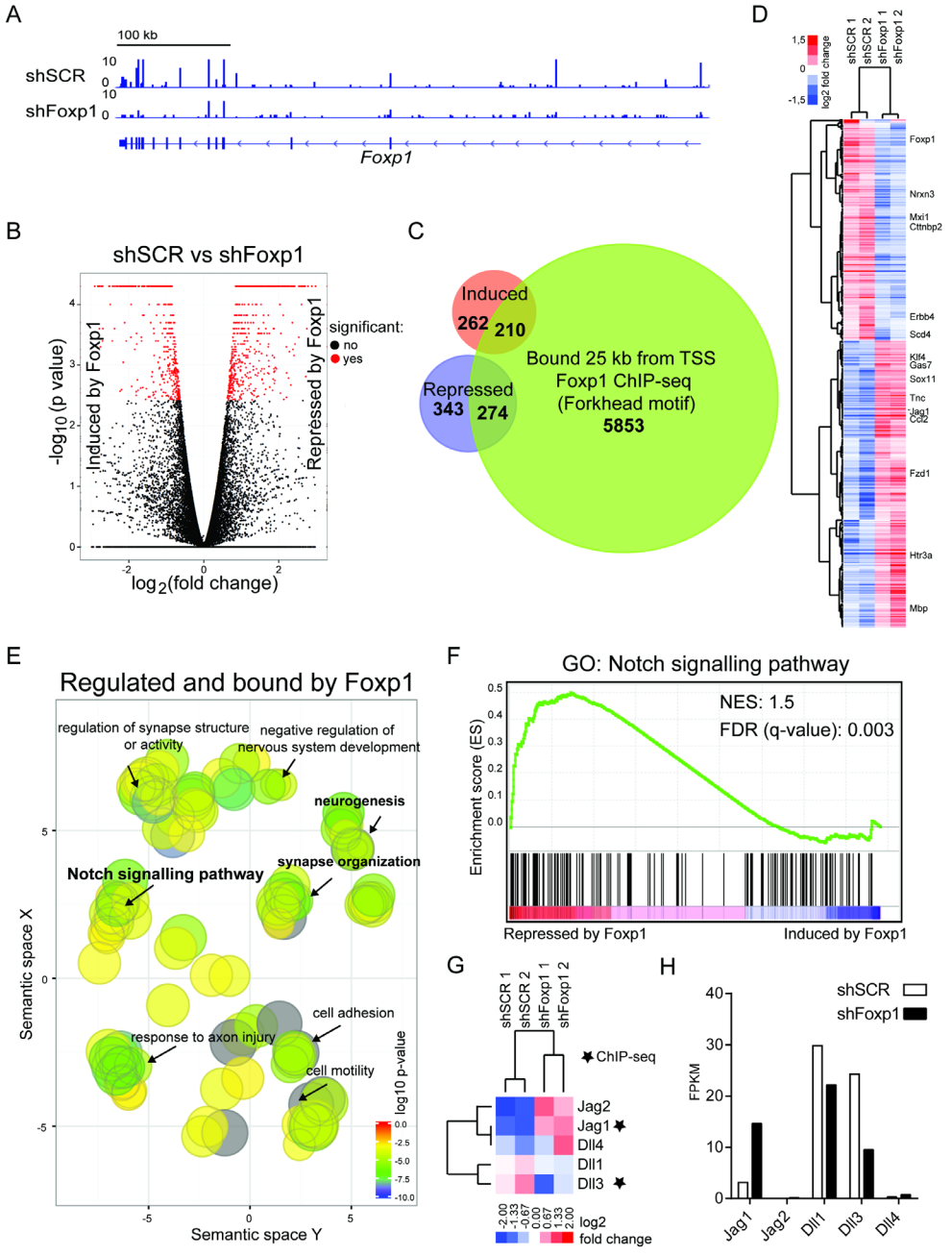


Figure 4. Genome-wide analysis of Foxp1 DNA-binding in NSCs.

ChIP-seq for Foxp1 was performed on chromatin isolated from cultured NSCs as described in Experimental Procedures. **(A)** Visualization of Foxp1 ChIP-seq profile within the genomic region surrounding the *Bik* loci. **(B)** Genomic distribution of Foxp1 binding sites in annotated regions compared to background genomic sites. **(C)** Average profile plot of Foxp1. RPM: reads per million. **(D)** Motif-distribution analysis of Foxp1 motif. **(E)** Motif enrichment analysis of Foxp1 using *de novo* motif discovery. **(F)** Gene ontology analysis using GREAT showing the genes associated with CNS diseases.

enrichment analysis (GSEA) probing for enrichment of genes belonging to the GO-term Notch signaling pathway in the RNA-seq dataset ranked by log fold-change. This analysis revealed a striking and significant enrichment of Notch signaling genes for genes repressed by Foxp1 in the RNA-seq dataset (**Figure 5F**). These data indicate that Foxp1 might directly repress one of the Notch ligands. Among the Notch ligands expressed in NSCs, Jagged 1 (Jag1), Jagged 2 (Jag2), Delta-like 1 (Dll1), Delta-like 3 (Dll3) and Delta-like 4(Dll4), only Dll3 and Jag1 were bound and differentially regulated by Foxp1 (**Figure 5G**). Dll3 was found to be induced by Foxp1 whilst Jag1 was found to be repressed by Foxp1 in the RNA-seq data (**Figures 5G and 5H**). Taken together our data indicate that Foxp1 regulates neurogenesis-specific genes and represses genes of the Notch pathway.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Induced by Foxp1
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significant:
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yes
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log2(fold change)
C
Induced
262
210
Repressed
343
274
Bound 25 kb from TSS
Foxp1 ChIP-seq
(Forkhead motif)
5853
D
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shSCR 2
shFoxp1 1
shFoxp1 2
log2 fold change
Foxp1
Nrxn3
Mxi1
Cttnbp2
Erbb4
Scd4
Klf4
Gas7
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Tnc
Jag1
Cdl2
Fzd1
Htr3a
Mbp
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regulation of synapse structure or activity
negative regulation of nervous system development
neurogenesis
synapse organization
Notch signalling pathway
cell adhesion
response to axon injury
cell motility
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Semantic space X
Semantic space Y
F
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NES: 1.5
FDR (q-value): 0.003
Enrichment score (ES)
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Induced by Foxp1
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shSCR 2
shFoxp1 1
shFoxp1 2
ChIP-seq
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Dll3
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H
shSCR
shFoxp1
FPKM
Jag1
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Dll4

Figure 5. Foxp1 regulates expression of neurogenesis related genes and inhibits Notch signaling pathway.

RNA-seq was performed on Foxp1 KD NSCs. Putative targets were identified by overlapping the expression data with the ChIP-seq dataset. **(A)** Visualization of RNA-seq reads around the genomic locus of *Foxp1* in control and KD conditions. **(B)** Volcano-plot representing differentially expressed genes in Foxp1 KD NSCs compared to control. **(C)** Venn-diagram showing overlap between Foxp1-bound and -regulated genes. **(D)** Heat-map showing the expression of Foxp1-bound and -regulated genes. **(E)** Gene ontology analysis using REVIGO showing the genes associated with neurogenesis and Notch signaling pathway. **(F)** Gene-set enrichment analysis (GSEA) representing the enrichment of Notch signaling pathway genes in the RNA-seq expression dataset ranked on log fold change after Foxp1 KD. **(G)** Heat-map showing the expression of Notch pathway ligands. **(H)** Plot of the average of the expression levels of Notch pathway ligands in the RNA-seq dataset. See also Figure S2.

Foxp1 promotes neurogenesis by transcriptionally repressing Jag1

To validate the regulation of the Notch ligands by Foxp1, the mRNA expression levels of Jag1, Dll3, Jag2, Dll4 and Dll1 were analyzed in NSCs expressing two independent Foxp1 shRNAs. Jag1 repression and Dll3 induction by Foxp1 were confirmed (**Figures 6B** and **S3A**). Dll3 is the least characterized of the Notch ligands, deemed to both inhibit Notch signaling in *cis* (Ladi *et al.,* 2005) and to activate the Notch pathway in the developing brain (Zhao *et al.,* 2009). Jag1, however, has been shown to be required for stem cell maintenance in the postnatal SVZ (Nyfeler *et al.,* 2005) and during granular cell neurogenesis in the adult dentate gyrus (Lavado and Oliver, 2014). Moreover Jag1 inhibits differentiation of adult neural stem cells by promoting NSC quiescence in the SVZ (Ottone *et al.,* 2014). This raises the possibility that Foxp1 promotes NSC differentiation by repressing Jag1 expression. Indeed, highly enriched regions for Foxp1-binding across the promoter region of Jag1 were identified (**Figure 6A**). The expression of Jag1 protein was also found to be increased in Foxp1-depleted NSCs after 5 days of differentiation when compared to control NSCs, suggesting that increased Jag1 levels inhibit differentiation (**Figures 6C-6D**). If Foxp1 indeed represses Jag1 expression during NSC differentiation, an increase in Foxp1 levels would correspond to a decrease in Jag1 expression. To evaluate this, Jag1 and Foxp1 mRNA expression were measured during NSC differentiation. When compared to undifferentiated NSCs, Jag1 mRNA was significantly reduced after 2 days of differentiation corresponding to an increase in Foxp1 mRNA (**Figure 6E**).

If an increase in Jag1 expression in Foxp1 KD NSCs is responsible for reduced NSC differentiation, blocking the interaction between Jag1 and Notch receptor would act to rescue this phenotype. Foxp1 KD NSCs were treated with an anti-Jag1 blocking antibody during 5 days of differentiation. As observed in the earlier experiments shown in **Figures 1E, 1G and 1H**, Foxp1 depletion reduced both βIII-tubulin- and GFAP-positive cells after differentiation (**Figures 6F, 6G and S3B**). Strikingly, upon treatment with the anti-Jag1 antibody, the number of βIII-tubulin-positive cells deriving from Foxp1 KD NSCs was restored to the level of controls, when compared to Foxp1-depleted NSCs treated with the isotype control (**Figures 6F and 6G**). However, no significant effect was observed in the number of GFAP-positive cells deriving from Foxp1 KD NSCs upon anti-Jag1 treatment (**Figure S3B**). This suggests that increased Jag1 expression in Foxp1 KD NSCs inhibits differentiation specifically towards neurons.

Taken together, these observations strongly indicate that Foxp1 represses Jag1 expression by binding to its promoter, and that this repression is essential for NSC differentiation.

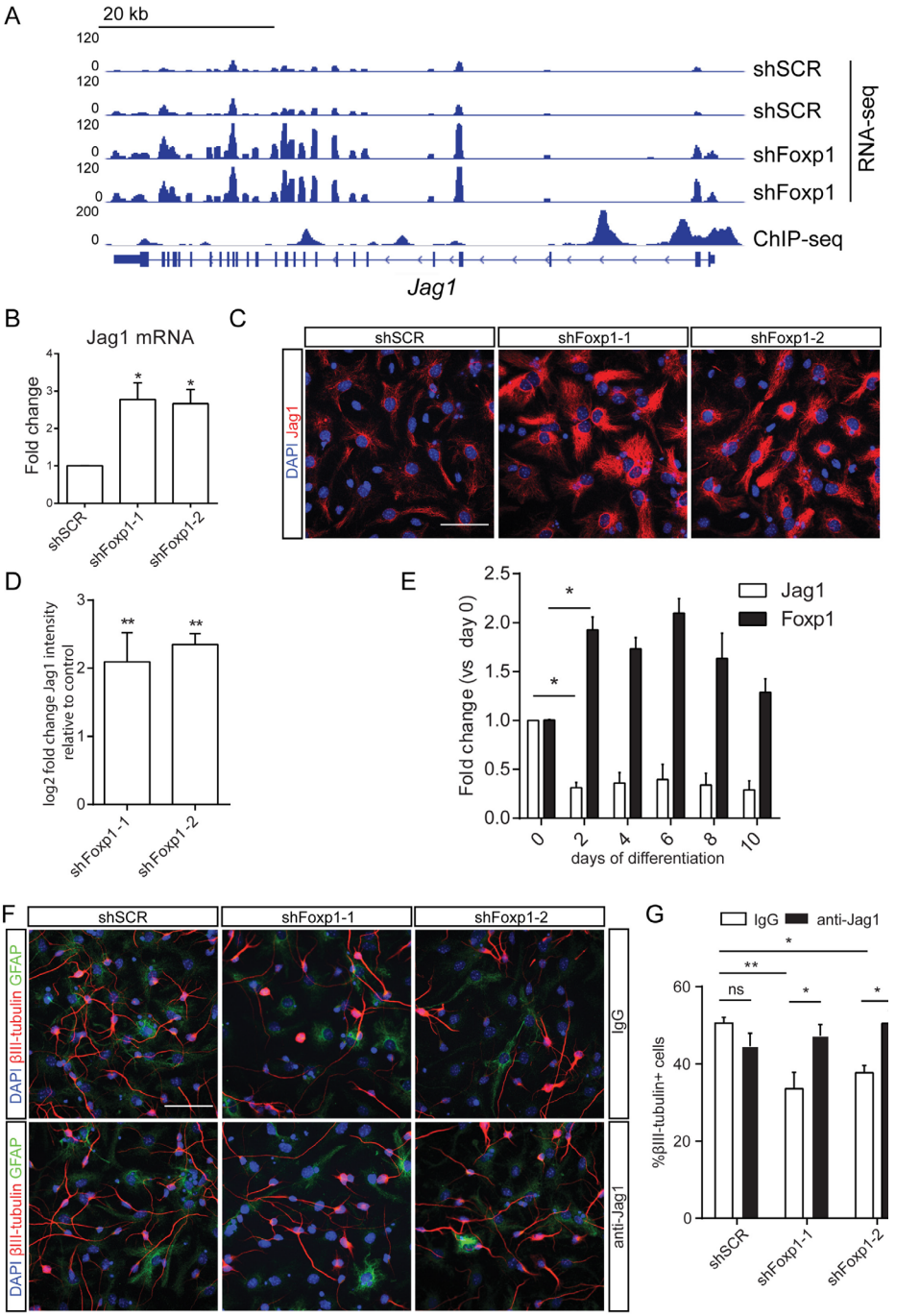

Figure 6. Foxp1-mediated expression of Jag1 is required for neuronal differentiation of NSCs.

(A) Visualization of RNA-seq reads and Foxp1 ChIP-seq profile around the genomic locus of Jag1 in control and KD conditions. **(B)** Measure of Jag1 mRNA levels upon Foxp1 KD (n=3) *=p<0.05. (C) Confocal images showing the expression of Jag1 after 5 days of differentiation (red). DAPI co-stained nuclei in blue (bar=50 µm). **(D)** Quantification of C (n=3) **=p<0.01. **(E)** NSCs were differentiated for 10 days. Measure of Foxp1 and Jag1 mRNA level (n=3). *=p<0.05. **(F)** Foxp1 KD NSCs were differentiated for 5 days in presence of the anti-Jag1 blocking antibody. Representative confocal images showing expression of the neuronal marker βIII-tubulin (red) and the astrocyte marker GFAP (green). DAPI co-stained nuclei in blue (bar=50 µm) **(G)** Quantification of F (n=3). *=p<0.05, **= p<0.01, ns= non-significant. Error bars represent SEM. See also Figure S3.

Discussion

Here we demonstrate for the first time that Foxp1 is a critical regulator of embryonic NSC differentiation based on several observations. Firstly, Foxp1 is required for *in vitro* differentiation of NSCs towards neurons and astrocytes, but is not involved in oligodendrocyte fate specification. Secondly, utilizing two disparate models, we show that Foxp1 regulates neuronal differentiation *in vivo*. Namely, we demonstrated that NSCs transplanted into the hippocampus of mice with HI brain injury require Foxp1 to become neuroblasts. Furthermore, Foxp1 in NSCs was essential after transplantation to conduct functional recovery in HI mice. Moreover we showed that Foxp1 was required for differentiation of neurons originating from the radial glia during embryonic corticogenesis. Lastly, we evaluate the underlying molecular mechanisms, showing how Foxp1 not only controls expression of multiple genes regulating neurogenesis, but specifically inhibits the Notch signaling pathway through direct transcriptional repression of Jag1. These novel data detail for the first time a key role for Foxp1 in regulating the functionality of NSCs, and define an underlying molecular mechanism involving regulation of the Notch pathway by targeting Jag1.

Recent evidence has suggested that Foxp1 is a major determinant in several neurodevelopmental diseases such as autism, speech defects, and intellectual disabilities, however so far this has lacked a detailed mechanistic explanation (Hamdan *et al.,* 2010; Le Fevre *et al.,* 2013; Lozano *et al.,* 2015; Palumbo *et al.,* 2013; Sollis *et al.,* 2016). Importantly, the specific cell types involved in the etiology of these conditions have not been characterized. We propose that Foxp1 has a crucial role in promoting embryonic NSC differentiation and migration. Whether Foxp1 KD is affecting both migration and differentiation of NSCs separately or whether reduced differentiation affects the migratory capacity of the NSCs from the VZ remains unclear. Defects in the regulation of neural progenitors during development are associated with a wide range of neurodevelopmental disorders, including autism (Kaushik and Zarbalis, 2016). To this end we found that Foxp1 binds near genes linked to CNS diseases. In accordance with a previous study, we observed that Foxp1 can regulate a subset of autism-related genes from the Simons Foundation Autism Research Initiative dataset (SFARI) (http://sfari.org) (Araujo *et al.,* 2015; Basu *et al.,* 2009) (**Figure S2A**). These observations indicate that these disease-associated genes might become deregulated during development due to mutations altering the functionality of Foxp1 or changes in its expression levels (Chien *et al.,* 2013; Lozano *et al.,* 2015). In our ChIP-seq analysis we

found the consensus motives of the neurodevelopmental transcription factors Sox3 and Nr2e1/TLX associated with the Forkhead motif (Archer *et al.,* 2011; Wang and Xiong, 2016), indicating a possible interaction between Foxp1 and these factors to regulate genes involved in neurogenesis. Sox3 inhibits neural progenitor differentiation by preventing premature activation of neuronal genes by competing for binding sites with Sox11 (Bergsland *et al.,* 2011). It is possible that Foxp1 may compete for binding of pro-neural genes with Sox3, thereby inducing differentiation. Similarly, Nr2e1/TLX controls the expression of a gene-network involved in NSC maintenance (Islam and Zhang, 2015) and Foxp1 may repress their transcription, thereby promoting neurogenesis. We have previously shown that Foxp1 expression can be transcriptionally induced by FoxO3, with Foxp1 subsequently inhibiting a subset of transcriptional targets activated by FoxO3 through a negative feedback loop (van Boxtel *et al.,* 2013). In NSCs, FoxO3 has been shown to be required for NSC homeostasis by inducing a program of genes that prevents premature differentiation (Renault *et al.,* 2009). Possibly, Foxp1 may be activated by FoxO3 also in NSCs where it may then suppress FoxO3-dependent genes that prevent differentiation.

The Notch pathway has a fundamental role in the maintenance of neural progenitors. After activation of the Notch receptor by its ligands, such as Jag1 or Dll1, the Notch intracellular domain (NICD) translocates to the nucleus. There, together with Rbpj, NICD induces the expression of transcriptional inhibitors like Hes1 and Hes5. These factors repress the expression of pro-neural genes, thereby preventing differentiation (Gaiano and Fishell, 2002; Kopan and Ilagan, 2009; Louvi and Artavanis-Tsakonas, 2006; Shimojo *et al.,* 2008). As our data indicate, Foxp1 binds directly to the Jag1 promoter, thereby repressing its transcription. When Foxp1 levels are reduced, the increased expression of Jag1 on the cell surface results in Notch activation in neighboring cells thereby preventing differentiation. We have shown that Foxp1-mediated Jag1 repression is a requirement for neuronal differentiation, but not for astrocyte specification *in vitro*. Therefore it will be relevant to investigate other Foxp1 targets involved in astrogliogenesis. Previously, it has been shown that expression of Jag1 by endothelial cells in the adult SVZ induces quiescence in NSCs by promoting the expression of stemness genes (Ottone *et al.,* 2014). In line with this, we observed increased expression of the neural stem/progenitor marker Nestin after Foxp1 knockdown *in vitro*. During NSC differentiation the expression of lineage-specific genes is increased with concomitant inhibition of processes involved in the maintenance of the stem cell/progenitor state such as the Notch pathway (Imayoshi *et al.,* 2013; Shimojo *et al.,* 2008). Strikingly, upon Foxp1 knockdown, we observed an induction of genes related to neurogenesis (**Figure S2B**). Taken together these observations indicate that Foxp1 acts by both inducing the expression of neurogenesis-specific genes as well as by directly repressing Jag1.

The detection of the expression of two isoforms of Foxp1, which share the same mRNA (Foxp1A and Foxp1C), raises the question whether there would be an isoform-specific role

for Foxp1. Foxp1C, the smaller isoform, is translated starting from a differential start codon and it lacks the Q-rich domain (Shu *et al.,* 2001). The shRNAs we utilized to deplete Foxp1 in our experiments target the mRNA that code for both isoforms. This makes it impossible to investigate the specific role of each isoform in regulating NSC differentiation. Foxp1 has also been shown to both homo- and heterodimerize with Foxp2 and Foxp4 (Shu *et al.,* 2001; Sin *et al.,* 2015). Notably, Foxp1, Foxp2 and Foxp4 expression is simultaneuosly found in different areas of the songbird and human brain (Chen *et al.,* 2013; Mendoza *et al.,* 2015; Teramitsu *et al.,* 2004). Moreover, Foxp1 and Foxp2 have been shown to regulate shared target in the murine striatum (Araujo *et al.,* 2015). It is possible that the Foxp1C isoform lacks the capacity of dimerization, therefore regulating transcription in a distinct manner to Foxp1A. Foxp1 has been mainly regarded as a transcriptional repressor (Shu *et al.,* 2001; van Boxtel *et al.,* 2013). However, in our own data we observed that the expression of the majority of Foxp1-bound genes were either repressed or activated (data not shown). It could be that in NSCs Foxp1 plays a role as both an inducer and a repressor of transcription, perhaps with one isoform of Foxp1 activating gene expression and the other one repressing it.

In conclusion, we demonstrate that Foxp1 promotes NSC differentiation both *in vitro* and *in vivo*, and that this occurs at least in part through the repression of Jag1 by Foxp1. This study sheds light on a novel regulator of neural development and identifies Foxp1 as a novel modulator of the Notch pathway. Identifying key regulators of NSC differentiation during development of the brain as well as after cerebral injury might provide future tools to develop novel treatments for neurodevelopmental disorders or neurological diseases that would benefit from enhanced neuroregeneration.

Experimental Procedures

qPCR

RNA was isolated using the RNAeasy kit (Qiagen, Hiden, Germany) according to manufacturers' instruction. Reverse transcriptase reaction for the generation of cDNA was performed using iScript cDNA synthesis kit (Bio-rad, Hercules, CA) followed by real-time quantification with the LightCycler 96 Real-Time PCR System (Roche Life Sciences, Penzberg, Germany) using SYBR Green Supermix (Bio-Rad) for cDNA application following manufacturers' protocol. Relative expression was calculated with the ΔΔCt method using beta-2-microtubulin (B2m) to normalize. The list of primers used can be found in Supplemental Experimental Procedures.

Western blot

Cells were lysed directly in the plate with Laemmli buffer (0.12 mol/L Tris-HCL (pH6.8), 4% SDS and 20% glycerol). Protein concentration was measured with the Lowry assay. 40 µg of each sample were analysed by Sodium dodecil sulfate polyacrilammide gel (SDS-PAGE) and transferred by electrophoresis onto polyvinylidene difluoride membrane (Millipore,

Bedford, MA). The membranes were blocked using 2% BSA in TBST (0.3% Tween, 10 mM Tris pH 8 and 150 mM NaCl in H_2O) and probed with anti-Foxp1 (Cell Signaling Technologies, Danvers, Massachusetts, #2005, 1:1000) and anti-Tubulin (Sigma Aldrich, St. Louis, MS, T5168, 1:50000). Signal was detected using Amersham ECL Western Blotting Detection Reagent (Little Chalfont, United Kingdom).

Animals

Transplantation of NSCs after hypoxic-ischemic brain damage

All experiments were performed in accordance to international guidelines and approved by Experimental Animal Committee Utrecht (DEC-Utrecht, University Utrecht, Utrecht, The Netherlands). For the transplantation experiment, 9 day old (P9) C57BL/6J mice underwent HI by permanent right carotid artery occlusion under isoflurane anesthesia (4% induction, 1.5% maintenance) followed by 45 min exposure to 10% oxygen (Nijboer *et al.,* 2008). This procedure results in unilateral damage to the hippocampus, neocortex, and striatum (van der Kooij *et al.,* 2010). Sham-operated control animals underwent anesthesia and incision only. In total 44 pups of both genders from 10 different litters were randomly distributed amongst all experimental groups. No significant gender differences were identified at any of the measured parameters. All analyses were performed in a blinded set-up.

At day 10 after induction of HI, $1x10^5$ NSCs resuspended in 2 µl PBS or vehicle (2 µl PBS) were injected under isoflurane anesthesia at 2 mm caudal to bregma, 1.5 mm right from midline, and 4 mm below dural surface in order to reach the ipsilateral hippocampus (Braccioli *et al.,* 2016). Part of the animals were sacrificed at day 15 (5 days post-treatment) after HI by overdose pentobarbital followed by transcardially perfusion with PBS followed by 4% FA. Brains were collected and post-fixed in 4% FA.

A part of the animals survived until day 28 after induction of HI and were subjected to the cylinder rearing test (CRT). The CRT was used to assess forelimb use asymmetry as described before (Schallert *et al.,* 2000; van der Kooij *et al.,* 2010). Briefly, mice were individually placed in a Plexiglas cylinder and observed for 3 minutes. The forepaw used to contact the cylinder wall during a weight-bearing full rear was scored as left (L; impaired), right (R; non-impaired), or both. Non-impaired (R) paw preference was calculated as follows: [(R−L)/(L+R+ both)] × 100% (van der Kooij *et al.,* 2010; van Velthoven *et al.,* 2010). Inclusion criterion was a minimum of 10 weight-bearing movements in 3 minutes. 2 animals from the shFoxp1-NSC group were excluded as they did not meet these inclusion criteria. The test was performed by a trained observer blinded to treatment.

In utero electroporation

For the in *utero* electroporation experiments mouse embryos were injected with combinations of shFoxp1-1 and shFoxp1-2 (1:1 molar ratio) or shSCR together with pCAG-

GFP. Motor cortices were targeted by electroporation with an ECM 830 Electro-Square-Porator (Harvard Apparatus, Holliston, MA) set to five unipolar pulses of 50 ms at 30 V (950-ms interval). Embryos were placed back into the abdomen, and abdominal muscles and skin were sutured separately. Embryos were collected at E17.5, and brains were fixed in 4% formaldehyde. For the analysis of the expression of Foxp1 in the developing cortex, embryos were collected at E12 and E14 and fixed in 4% FA.

Chromatin Immunoprecipitation and sequencing

Before the Chromatin Immunoprecipitation (ChIP) and sequencing, NSCs were grown as neurospheres in complete medium in 6-wells. ChIP was performed as previously described (van Boxtel *et al.,* 2013). Briefly, crosslink was performed with disuccinimidyl glutarate (DSG) (Thermo Scientifis) for 45 minutes followed by 30 minutes incubation with formaldehyde 1%. The reaction was blocked with glycine 100 mM. Shearing was performed using Covaris S2 (Covaris, Woburn, MA) for 8 min at maximum intensity. The sonicated chromatin was incubated O/N at 4°C in presence of 5µg of anti-Foxp1 (ab16645, Abcam) coupled to A/G sepharose beads (Santa Cruz Biotechnology). Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, MA) was used for End-repair, A-tailing and ligation of sequence adaptors. Samples were amplified by PCR and the libraries were size-selected in the 200-500 bp range. Bar-coded libraries were sequenced on Illumina NextSeq500 sequencer as previously described (Peeters *et al.,* 2015) (50bp, single-end, Utrecht sequencing facility, Utrecht Medical Center, Utrecht, The Netherlands).

RNA sequencing

Total RNA was extracted from adherent NSCs cultured on 6-well coated plastic plates for 48h using the RNAeasy Kit (Qiagen). RNA quality was tested on the Bioanalyzer (Agilent, Santa Clara, CA) and sample quality was optimal with RNA integrity number (RIN) higher than 9.0. Sample preparation was performed using Poly(A)Purist MAG Kit (Thermo Scientific) according to manufacturers' instructions. Isolated mRNA was subsequently repurified using mRNA-ONLY Eukaryotic mRNA Isolation Kit (Epicentre (Illumina, Inc.), Madison, WI, USA). Sequencing libraries were prepared using SOLiD Total RNA-Seq Kit (Applied Biosystems Life Technologies) according to the standard protocol recommendations and sequenced on SOLiD Wildfire sequencer to produce 50bp reads as previously described (van Boxtel *et al.,* 2013).

Gene Set Enrichment Analysis

For Gene Set Enrichment Analysis (GSEA) pre-ranked analysis was performed with the GSEA software probing for enrichment of mouse genes belonging to the GO-term Notch signaling pathway (GO:0007219) in the RNA-seq dataset ranked by log fold-change (Carbon *et al.,* 2009; Subramanian *et al.,* 2005).

Statistics

Data are expressed as mean ± SEM and regarded statistically significant if $p < 0.05$. Data were analyzed using one-way ANOVA with Dunnett's post-test. One-way ANOVA with Tukey's post-test was used for the cylinder rearing test. For the anti-Jag1 blocking antibody experiment two-way ANOVA with Sidak's and Tukey's post-test were used. For the analysis of GFP-positive cells in the embryos and DCX-DsRed-positive cells in the transplantation experiment, two-tailed, unpaired Student's t-test was used.

For additional information see Supplemental Experimental Procedures.

Author contributions

LB and CHN performed the experiments, analyzed the data. LB, CHN and PJC designed the experiments and wrote the manuscript. SV contributed to the NGS analysis. YA and RJP contributed with the in utero electroporation experiments. CJH provided intellectual input.

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Supplementary information

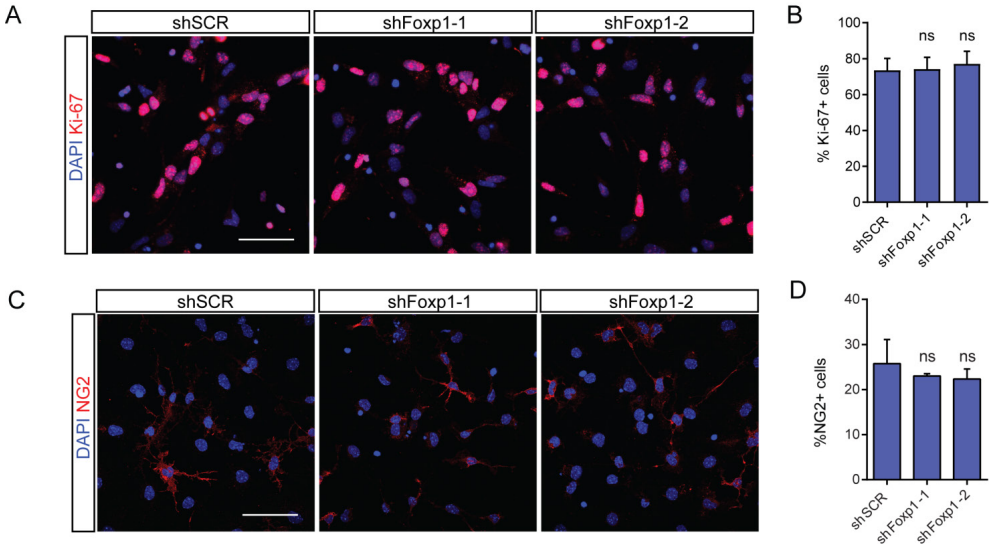

Figure S1. Related to Figure 1. Foxp1 does not regulate NSC proliferation and oligodendrocyte differentiation.

(A). NSCs were cultured in adhesion with growth factors for 48 hours and stained for Ki67 to assess proliferation. Representative confocal image showing expression of Ki67 (red), DAPI co-stained nuclei in blue (bar=50 µm). **(B)** Quantification of A. (n=3) ns = non significant. **(C)** Foxp1 KD NSCs were differentiated for 5 days. Representative confocal images showing the expression of the oligodendrocyte marker NG2 (red). DAPI co-stained nuclei in blue (bar=50 µm). **(D)** Quantification of S1C. (n=3) ns = non significant. Error bars represent SEM.

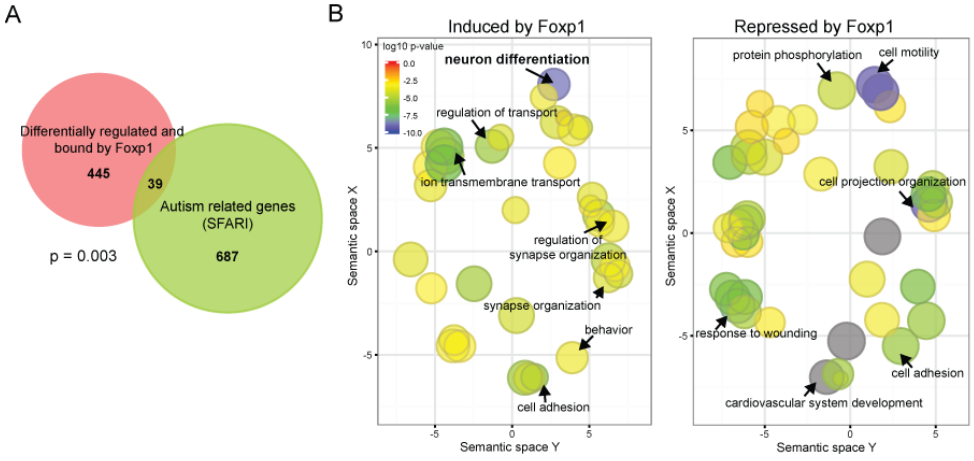

Figure S2. Related to Figure 5. Characterization of Foxp1-bound and -regulated genes.

(A) Venn-diagram showing overlap between Foxp1-bound and –regulated genes and austism related genes from the SFARI dataset. **(B)** Gene ontology analysis of Foxp1-bound, both -repressed or induced genes, using REVIGO showing the genes associated with biological functions.

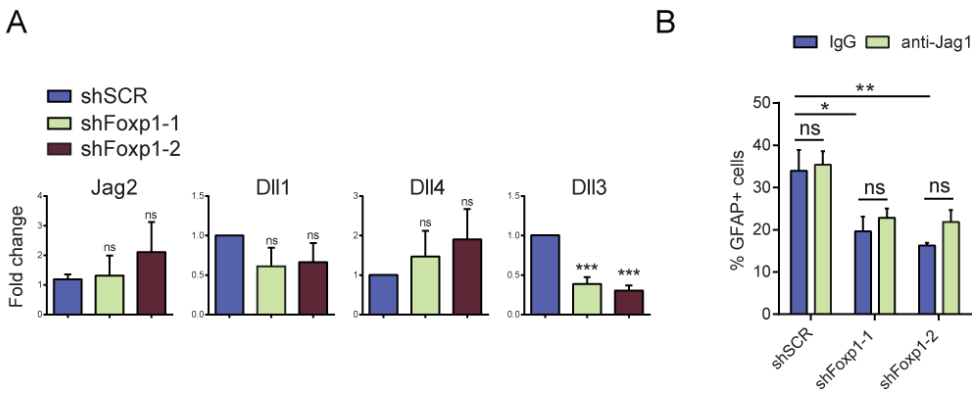

Figure S3. Related to Figure 6. Analysis of the expression levels of Notch ligands upon Foxp1 KD. Measurement of astrocyte differentiation upon treatment of Foxp1 KD NSCs with anti-Jag1 blocking antibody.

(A) Measurement of Jag2, Dll1, Dll3 and Dll4 mRNA expression levels (n=3). ***= p<0.001, ns= non significant. **(B)** Foxp1 KD NSCs were differentiated for 5 days in presence of the anti-Jag1 blocking antibody. Quantification of the number of astrocytes (n=3). *=p<0.05, **= p<0.01, ns= non significant. Error bars represent SEM.

Supplemental Experimental Procedures

List of qPCR primers used

Primer	Sequence
FW-Foxp1	AGGCTGTGAGGCGGTTTGT
REV-Foxp1	CATTGAGCTGTGCTTCTATCG
FW-Jag1	CATCGATTACTGCGAGCCCA
REV-Jag1	GCAGCTGTCAATCACTTCGC
FW-Jag2	GGGTGGCAACTCCTTCTACC
REV-Jag2	AGCTCCTCATCTGGAGTGGT
FW-Dll1	ACCAAGTGCCAGTCACAGAG
REV-Dll1	TCCATCTTACACCTCAGTCGC
FW-Dll3	GGGCAGCTGTAGTGAAACCT
REV-Dll3	CTTCACCGCCAACACACAAG
FW-Dll4	AGTGGACTGTGGTCTGGACA
REV-Dll4	GACACTCTGGCTTCTCACTGT
FW-B2m	CGCTCGTTGCCAATAGTGAT
REV-B2m	CGTGCGTGACATCAAAGAGA

FW: Forward primer

REV: Reverse primer

Cell culture

NSCs isolated from embryonic day 14.5 CD-1 mice were obtained from R&D Systems, Minneapolis, MN. NSCs were cultured as neurospheres in DMEM:F12 medium (Thermo Scientific, Waltham, MA) with 50x B-27 (Thermo scientific) plus 20 ng/mL EGF and 20 ng/mL bFGF (both Peprotech, Rocky Hill, NJ) and antibiotics (Thermo Scientific). For experiments NSCs were cultured onto 35 mm glasses coated with poli-L-ornithine (10µ/mL, Sigma Aldrich) and Laminin (1µ/mL, Sigma Aldrich) and let adhere for 48h. For the proliferation experiment cells were then fixed in formaldehyde 4%. For the differentiation assay, NSC differentiation was induced by growth factor withdrawal. Cells were fixed 5 days after in formaldehyde (FA) 4%. For the transplantation assay, neurospheres were dissociated into single cells and resuspended in PBS. Lentiviral particles were generated as previously described (Vervoort et al., 2013). NSCs were transduced twice in adherence on plates coated with poli-L-ornithine (10µ/mL) Sigma Aldrich) and Laminin (5µ/mL, Sigma Aldrich). The following shRNAs vectors were used: shFoxp1-1 (pLKO.1_sh_ex15, gift from Benjamin Blencowe, Addgene plasmid # 35166) (Gabut *et al*., 2011), shFoxp1-2 (TRCN0000072005, Sigma Aldrich), shSCR (SHC002, Sigma Aldrich). The cells were selected with puromycin (0.5 µg/mL). For the overexpression experiment the coding region murine of Foxp1 was amplified from pcDNA3.1 Foxp1A (gift from Anjana Rao, Addgene plasmid # 16362) (Hu *et al*., 2006) and cloned into the lentiviral vector pLX303 (gift from David Root, Addgene plasmid # 25897) (Yang *et al*., 2011). After transduction the cells were selected with Blasticidin (2µg/mL).

For the anti-Jag1 blocking antibody experiment cells were treated with 1 µg/mL of either hamster anti-Jag1 (16-3391-82, affymetrix eBioscience, San Diego, CA) or hamster IgG isotype control (16-4888-81, affymetrix eBioscience) every other day.

For in vivo tracing, NSCs were transduced with the lentiviral vector PhAGE2-EF1aFull-DsredExpress-IRES-NeoR-LoxP and selected with G418 (200 µg/mL).

Immunofluorescence

For in vitro experiments, cells were fixed in 4% FA and stained with rabbit anti-βIII-tubulin (Abcam, Cambridge, United Kingdom, 1:1000), mouse anti-GFAP (Acris antibodies, San Diego, CA, 1:50), rabbit anti-Ki67 (Abcam, Cambridge, United Kingdom, 1:500), rabbit anti-NG2 (Merck Millipore, Billerica, MA, 1:200), rabbit anti-Jag1 (gift from Verdon Taylor, 1:2000) (Nyfeler *et al*., 2005), mouse anti-Nestin (Becton Dickinson, Franklin Lakes, NJ, 1:200) followed by incubation with donkey anti-rabbit AlexaFluor-555 or goat anti-mouse AlexaFluor-488 (both Thermo Scientifics, 1:250). Nuclei were counterstained with 4',6-Diamidino-2-Phenylindole (DAPI). The expression level of Nestin and Jag1 per cell was determined using ImageJ software (Schneider *et al*., 2012). For the transplantation experiment brains were collected at 5 days post-HI, post-fixed, cryoprotected in 30% sucrose and embedded in OCT freezing medium (Tissue-Tec, Sajura Zoeterwoude, The Netherlands).

Coronal cryosections (20 µm) were incubated with rabbit anti-RFP (Abcam, 1:500), goat anti-DCX (Santa Cruz Biotechnology, Dallas, TX, 1:100) followed by incubation with secondary antibodies donkey anti-rabbit AlexaFluor555 or donkey anti-goat AlexaFluor488 (all Thermo Scientific, 1:250). Nuclei were counterstained with DAPI and sections were mounted with FluorSave reagent (Merck Millipore, Darmstadt, Germany). For the embryonic cortex analyses brains were collected, post-fixed in 4% FA for 3 hours, cryoprotected in 30% sucrose and embedded in OCT freezing medium. Coronal or sagittal cryosections (30 µm) were incubated with rabbit anti-Foxp1 (Cell Signalling Technologies, 1:400), goat anti-Sox2 (Santa Cruz Biotechnology, 1:500), rat anti-Ctip2 (1:200, Abcam) or goat-anti-GFP (Abcam, 1:500) followed by incubation with secondary antibodies donkey anti-rabbit AlexaFluor555, donkey anti-rat AlexaFluor594 or donkey anti-goat AlexaFluor488 (all Thermo Scientific, 1:250). Nuclei were counterstained with DAPI and sections were mounted with FluorSave reagent (Merck Millipore). Fluorescent confocal images were captured with an AxioCam MRm (Carl Zeiss, Sliedrecht, The Netherlands) on a LSM 700 confocal microscope (Carl Zeiss). The number of DCX and DsRed positive cells were quantified by counting cells in 3 different fields of the hippocampus. The number of GFP-positive cells in the embryo cortex was determined by counting cells in 3 consecutive sections caudally from the corpus callosum.

NGS data analysis

For RNA-sequencing analysis sequencing reads were mapped against the reference genome (mm10 assembly) using TopHat v2.0.9 as previously described (Trapnell *et al*., 2012). Only uniquely mapped reads were selected for further analysis. In brief, guide transcripts were assembled using CuffLinks v2.2.1. Reads were quartile normalized using the –library-norm-method quartile option. Differential gene analysis was performed using CuffDiff. Cluster 3.0 and Java TreeView software v1.1.6 were used for visualization of heatmaps. ChIP-sequencing reads were mapped with Bowtie 2.1.0 against the reference genome (mm10) using default settings (Langmead and Salzberg, 2012). Peaks were called with HOMER software using the input as a control (Fold over local region=8, Fold over input=2) (Heinz *et al*., 2010). Mapped fragments were extended according to the average fragment size and converted to TDF files, visualized with IGV tools v2.3.36 and represented as coverage normalized tracks (Robinson *et al*., 2011). SAMtools was used for manipulation of SAM and BAM files; manipulation of BED file format was performed with BEDtools (Li *et al*., 2009; Quinlan and Hall, 2010). Motif discovery, peak annotation and generation of histograms were performed using HOMER software. For motif discovery the 200 bp sequence surrounding each peak was examined and motifs with length of 8, 10 and 12 nt were searched. Motifs were identified through de novo motif discovery. For functional annotation of peaks the software GREAT was used, where genes that presented peaks within 1kb with Forkhead motif were selected (McLean *et al*., 2010). For the selection of Foxp1 target genes, genes that presented a peak with Forkhead motif within 25kb were selected. The ToppGene suite database (https://toppgene.cchmc.org/enrichment.jsp) and the REVIGO visualization tool were used for GO-term analysis of

differentially expressed and Foxp1-bound genes (Chen *et al*., 2009; Supek *et al*., 2011). For analysis of Autism-associated genes, the human genes belonging to the category Autism and Autism Spectrum Diseases of the SFARI database (https://gene.sfari.org) were converted to mouse identifiers and overlapped with the differentially expressed and Foxp1-bound genes (Basu *et al*., 2009); statistical analysis was performed with the Hypergeometric test.

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CHAPTER 3

Sox4 inhibits oligodendrocyte differentiation of embryonic neural stem cells by inducing Hes5 expression.

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In preparation.

Abstract

Sox4 has been shown to promote neuronal differentiation both in the adult and embryonic neural progenitors. Ectopic Sox4 expression has been shown to inhibit oligodendrocyte specification in mice, however the underlying molecular mechanism remains poorly understood. Here we demonstrate that Sox4 regulates transcriptional targets associated with neural development in neural stem cells (NSCs), reducing the expression of genes promoting oligodendrocyte differentiation. Accordingly, we show that Sox4 knockdown induces increased oligodendrocyte differentiation, as the percentage of Olig2-positive/2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase)-positive maturing oligodendrocytes increases, while the number of Olig2-positive oligodendrocyte precursors is unaffected. Conversely, conditional Sox4 overexpression utilizing a doxycycline inducible system decreases the percentage of maturing oligodendrocytes, suggesting that Sox4 inhibits maturation from precursor to mature oligodendrocyte. Finally we identify Hes5 as a Sox4 target gene. Since Hes5 is a transcription factor described to inhibit oligodendrocyte maturation and myelination, it could likely mediate inhibition of oligodendrocyte differentiation by Sox4. Taken together, these observations show a novel role for Sox4 in NSC by controlling oligodendrocyte differentiation.

Introduction

Neural stem cells (NSCs) are multipotent progenitor cells residing throughout both the developing and the adult brain in specialized niches of the subventricular zone (SVZ) and the subgranular layer (SGL) of the hippocampal dentate gyrus (Alvarez-Buylla et al., 2001). During corticogenesis, the radial glia, which are considered embryonic NSCs, locate at the ventricular zone (VZ) and differentiate into neurons, astrocytes and oligodendrocytes while migrating toward the cortical plate (CP) (Fishell and Kriegstein, 2003; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). Growing evidence demonstrates that NSCs transplantation shows potential in treating pathological conditions of the central nervous system (CNS) such as hypoxic-ischemic encephalopathy, multiple sclerosis, Alzheimer's disease and cerebral stroke (Bacigaluppi et al., 2016; Blurton-Jones et al., 2009; Braccioli et al., 2016; Daadi et al., 2010; Martino and Pluchino, 2006; Park et al., 2002). NSC differentiation is a finely tuned process regulated amongst others by the Notch pathway, which is involved not only in neural progenitor maintenance in the adult and embryonic compartment, but also in fate decisions and lineage commitment (Ables et al., 2011; Louvi and Artavanis-Tsakonas, 2006). For instance, the Notch target gene Hes5, a basic-helix-loop-helix transcriptional repressor, has been described as negatively regulating neural and oligodendrocyte differentiation while promoting NSC maintenance (Kondo and Raff, 2000; Liu et al., 2006; Ohtsuka et al., 1999; Ohtsuka et al., 2001).

Sox4 is a transcription factor belonging to SoxC group of the SRY-related HMG-box (SOX) family, which are important for embryonic development and tissue homeostasis, as well as cancer progression (Vervoort et al., 2013b; Wegner, 2010). The SoxC family consists of Sox4, Sox11 and Sox12, which share a high level of identity within the high-mobility group (HMG) domain and the C-terminal transactivation domain (TAD) (Dy et al., 2008). Sox4 expression overlaps with other SoxC factors in several compartments during embryogenesis, including mesenchymal tissue, peripheral and central nervous system (Vervoort et al., 2013b). During chick development, Sox4 and Sox11 have been shown to induce the expression of neuronal genes such as βIII-tubulin and MAP2, suggesting they may regulate the late stages of neuronal differentiation (Bergsland et al., 2006). Additionally, Sox4 and Sox11 promote neural progenitor proliferation and survival (Bhattaram et al., 2010). Moreover, Mu and colleagues demonstrated that Sox4 and Sox11 promote neurogenesis in the SGL by activating doublecortin (Dcx) expression (Mu et al., 2012). Although highly homologous, Sox4 and Sox11 have been show to play *non*-redundant roles by regulating separate population of neurons during corticogenesis: Sox11 interacts with Neurogenin1 in early born neurons hence activating the pro-neural gene NeuroD1, while Sox4 is expressed by intermediate progenitor cells and promotes their maintenance by interacting with Neurgenin2 to activate Tbrain2 (Chen et al., 2015). Sox4 has also been reported to regulate gliogenesis, as prolonged expression of a Sox4 transgene under the control of the human GFAP promoter activating transgene expression in radial glia and astrocytes, leads to defects in the morphology

of the cerebellum and ataxia in mice (Hoser et al., 2007). Moreover, the expression of a Sox4 transgene induced by the myelin basic protein (MBP) promoter, which is active in differentiting oligodendryocytes, impairs myelination in the whole CNS (Potzner et al., 2007). Accordingly, it has been shown that ectopic expression of Sox4 in Schwann cells by the myelin protein zero (MPZ) gene and the enhancer of MBP inhibits myelination and worsens neuropathic phenotype in a mouse model of Charcot–Marie–Tooth 4C disease (Bartesaghi et al., 2015). Taken together these observations suggest that Sox4 negatively regulates glial differentiation, however the underlying mechanisms regulated by Sox4 remains unknown. To this end we explored the role of Sox4 during embryonic NSC differentiation. We combined global transcriptional profiling with *in vitro* analyzes, identifying Sox4 as a negative regulator of oligodendrocyte differentiation. Finally, we identify Hes5 as a target of Sox4, which may mediate Sox4 repression of NSC oligodendrocyte differentiation. Taken together these findings indicate a unique role for Sox4 in regulating oligodendrocyte differentiation of NSCs.

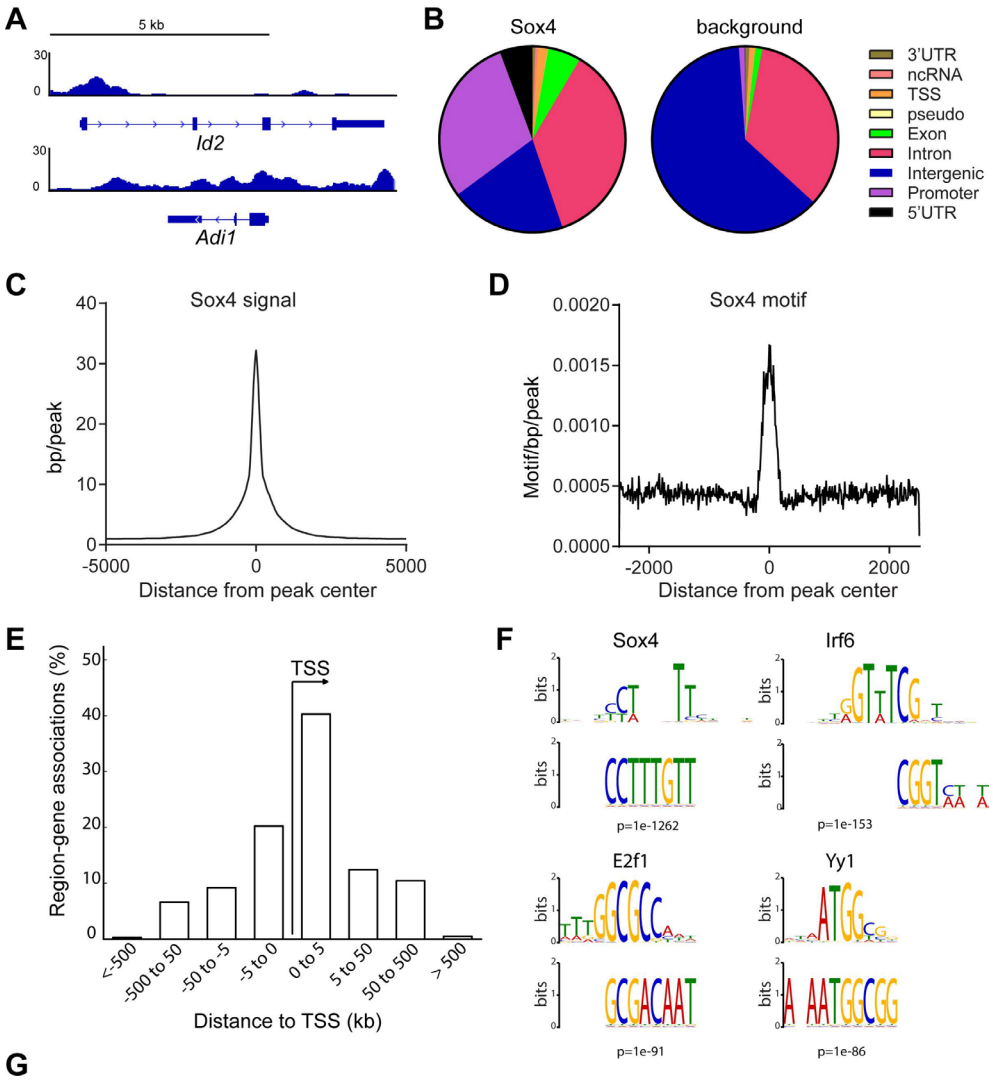
Results

Genome-wide analysis of Sox4 DNA-binding in NSCs

Sox4, together with its homologous Sox11, has been previously described to promote differentiation of both embryonic and neural progenitor differentiation (Bergsland M *et al,* 2006, Mu L *et al,* 2012). However, due to the high redundancy between Sox4 and Sox11, the role of Sox4 alone in the regulation of embryonic NSC biology has not been fully elucidated. In order to determine the specific function of Sox4 in NSC, we first investigated the genome-wide binding of Sox4 through chromatin-immunoprecipitation combined with sequencing (ChIP-seq). Sox4-bound regions were effectively detected as exemplified by peaks mapped at the region of the genes *Id2* and *Adi1* (**Figure 1A**). Analysis of the distribution of Sox4-binding events revealed an enrichment for Sox4-binding in promoter regions and 5'-untranslated regions (5'-UTR) when compared to random genomic sequences (**Figure 1B**). Moreover, Sox4-signal was enriched around the center of the peaks, thus validating the peak-calling procedure (**Figure 1C**). Additionally, Sox4-motif was found enriched at the center of the peaks, hence confirming sequence-specific binding (**Figure 1D**). Furthermore, Sox4-binding was found enriched around TSS (**Figure 1E**). *De novo* motif analysis identified Sox4-motif as the most enriched motif, followed by the binding-motives of the transcription factors Interferon Regulatory Factor 6 (Irf6), E2F Transcription Factor 1 (E2F1) and Yy1 (**Figure 1F**). To identify the genes that are neighboring Sox4-bound regions, the genes whose TSS was within 5 kilobase (kb) from peaks were further selected for gene-ontology (GO) analysis. Sox4-bound genes showed a significant association with abnormality of the brain, morphological abnormality of the central nervous system and cognitive impairment (**Figure 1G**). These observations are in agreement with a previous report indicating that transgenic Sox4 expression within the CNS, mediated by the glial fibrillary acidic protein (GFAP) promoter, induces architectural cerebellar defects and ataxia (Hoser M *et al,* 2007) . Taken together, these data show how Sox4 is binding to promoter regions of genes related to abnormalities of the CNS.

Sox4 regulates neurodevelopment associated genes in NSCs

In order to identify the genes modulated by Sox4, we first generated short-hairpin RNAs (shRNA)-mediated Sox4 knockdown (KD) NSCs. Analysis of Sox4 mRNA and protein expression revealed efficient KD (**Figure 1A and 1B**). Next, we performed RNA-sequencing (RNA-seq) on Sox4 KD NSCs which confirmed successful knockdown of Sox4 mRNA (**Figure 1C**). We confimed the specificity of the shRNAs targeting Sox4 by analyzing the expression of the other members of the SoxC family: both Sox11 and Sox12 were not found reduced in the RNA-seq data upon Sox4 KD (**Figure S1A**). Analysis of RNA-seq data showed that 389 genes were significantly induced and 226 genes were significantly repressed by Sox4 (**Figure 2D and 2E**). To identify the genes that are possibly regulated by Sox4 in a direct manner in NSCs, the set of differentially expressed genes was overlapped with the set of genes whose



G

	-log10(binomial p-value)
Abnormality of the brain	0.000
Morphological abnormality of the central nervous system	0.000
Cognitive impairment	0.000
Abnormality of higher mental function	0.000
Abnormality of muscle physiology	0.000
Abnormality of the central nervous system	0.000
Abnormality of the musculature	0.000
Abnormality of the nervous system	0.000

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Figure 1. Genome-wide analysis of Sox4 chromatin-binding in NSCs.

ChIP-seq for Sox4 was performed on chromatin isolated from cultured NSCs as described in Methods. **(A)** Visualization of Sox4 ChIP-seq profile within the genomic region surrounding the *Id2* and *Adi1* loci. **(B)** Genomic distribution of Sox4 binding sites in annotated regions compared to background genomic sites. **(C)** Average signal plot of Sox4. **(D)** Motif-distribution analysis of Sox4 motif. **(E)** Average distance to TSS plot of Sox4 peaks **(F)** Motif enrichment analysis of Sox4 using *de novo* motif discovery. **(G)** Gene ontology analysis using GREAT showing the genes associated with CNS abnormalities.

TSS is within 5 kb from peaks derived from the ChIP-seq analysis. Of the Sox4-modulated genes, 136 were found to be both induced and bound by Sox4, whilst 130 were found to be repressed and bound by Sox4 (**Figure 2E and 2F**). To identify which biological processes were potentially regulated by Sox4, we performed gene ontology (GO)-term analysis using the set of Sox4-bound and regulated target genes. We observed the most significant association with CNS development; furthermore we observed a significant association with Notch signaling pathway (**Figure 2G**). In order to outline which CNS development processes were affected by Sox4, we performed a weighted gene-set enrichment analysis (GSEA) in the RNA-seq dataset ranked by log-fold change. This analysis revealed a significant enrichment of genes that are induced during oligodendrocyte differentiation for genes repressed by Sox4 in the RNA-seq dataset (**Figure 2H**). Strikingly, we observed both a significant enrichment of genes associated with a pro-neural phenotype in glioblastoma and of astrocyte marker genes for genes induced by Sox4 in the RNA-seq dataset (**Figure 2I and 2J**). Taken together, these data indicate that Sox4 regulated a subset of genes involved in CNS development and Notch signaling; moreover these observation suggest that Sox4 represses genes that are involved in oligodendrogenesis while inducing genes associated with neural and astrocyte differentiation. These findings are in line with reports showing Sox4 and Sox11 as promoting neural in adult NSCs (Mu et al., 2012).

Sox4 inhibits oligodendrocyte differentiation of NSCs

Since Sox4 repressed genes involved in oligodendrogenesis in our RNA-seq dataset, we wished to determine whether modulation of Sox4 expression altered oligodendrocyte differentiation in NSCs. To this end we differentiated Sox4 KD NSCs toward oligodendrocytes by removing basic-fibroblast growth factor (bFGF) and epidermal growth factor (EGF) and supplementing triiodothyronine (T3) to the differentiation medium (Baas et al., 1997). After 5 days of differentiation, Sox4 KD increased the percentage of 2′–3′ cyclic nucleotide 3′phosphodiesterase (CNPase)+-oligodendrocytes, whilst the percentage of Olig2+-oligodendrocyte precursor cells remained unaffected when compared to control (**Figure 3A-3C**) . This suggest that Sox4 inhibits the maturation of NSCs from Olig2+-precursor cells into CNPase+-cells during oligodendrocyte differentiation. In order to investigate whether ectopic Sox4 expression would then impair oligodendrocyte differentiation, Sox4 was ectopically expressed in NSCs utilizing a doxycycline (dox)-inducible system. Upon 24 h dox treatment, Sox4 overexpression (OE) was clearly detected, but not in the control NSCs (**Figure 3D**). Upon 5 days of differentiation, Sox4 expression reduced the percentage of CNPase+ oligodendrocytes, whilst the percentage of Olig2+-oligodendrocyte precursor

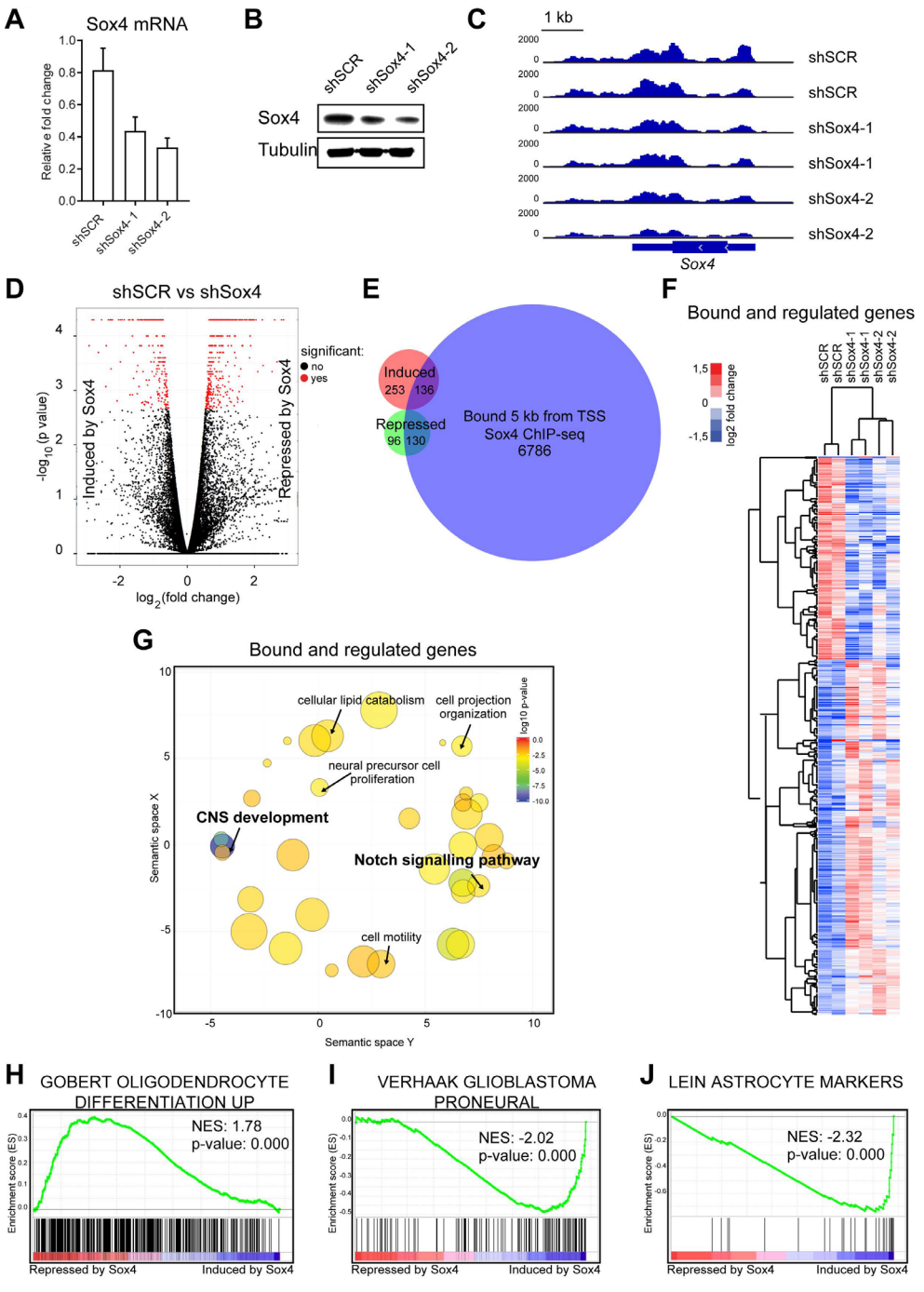
A
Sox4 mRNA
Relative fold change
shSCR
shSox4-1
shSox4-2
B
shSCR
shSox4-1
shSox4-2
Sox4
Tubulin
C
1 kb
shSCR
shSCR
shSox4-1
shSox4-1
shSox4-2
shSox4-2
Sox4
D
shSCR vs shSox4
significant:
no
yes
Induced by Sox4
Repressed by Sox4
-log10 (p value)
log2(fold change)
E
Induced
253 136
Repressed
96 130
Bound 5 kb from TSS
Sox4 ChIP-seq
6786
F
Bound and regulated genes
log2 fold change
shSCR
shSCR
shSox4-1
shSox4-1
shSox4-2
shSox4-2
G
Bound and regulated genes
cellular lipid catabolism
cell projection organization
neural precursor cell proliferation
CNS development
Notch signalling pathway
cell motility
log10 p-value
Semantic space X
Semantic space Y
H
GOBERT OLIGODENDROCYTE DIFFERENTIATION UP
NES: 1.78
p-value: 0.000
Enrichment score (ES)
Repressed by Sox4
Induced by Sox4
I
VERHAAK GLIOBLASTOMA PRONEURAL
NES: -2.02
p-value: 0.000
Enrichment score (ES)
Repressed by Sox4
Induced by Sox4
J
LEIN ASTROCYTE MARKERS
NES: -2.32
p-value: 0.000
Enrichment score (ES)
Repressed by Sox4
Induced by Sox4

Figure 2. Sox4 regulates expression of CNS development related genes and represses oligodendrocyte differentiation genes.

RNA-seq was performed on Sox4 KD NSCs. Putative targets were identified by overlapping the expression data with the ChIP-seq dataset. **(A)** Measure of Sox4 mRNA levels upon Sox4 KD with 2 independent shRNAs (n=3). **(B)** Representative western blot showing efficient knockdown of Sox4. **(C)** Visualization of RNA-seq reads around the genomic locus of *Sox4* in control and KD conditions. **(D)** Volcano-plot representing differentially expressed genes in Sox4 KD NSCs compared to control. **(E)** Venn-diagram showing overlap between Sox4-bound and -regulated genes. **(F)** Heat-map showing the expression of Sox4-bound and -regulated genes. **(G)** Gene ontology analysis using REVIGO showing the genes associated with CNS development and Notch signaling pathway. **(H)** Gene-set enrichment analysis (GSEA) representing the enrichment of oligodendrocyte differentiation genes in the RNA-seq expression dataset ranked on log fold change after Sox4 KD. (**I)** GSEA representing the negative enrichment of pro-neural and **(J)** astrocyte differentiation genes in the RNA-seq expression dataset ranked on log fold change after Sox4 KD.

cells remained unaltered when compared to control (**Figure 3E-3G**). These data confirm that Sox4 represses the differentiation of Olig2+-precursor cells into CNPase+-cells during oligodendrocyte differentiation. Taken together, these observations show that Sox4 inhibits oligodendrocyte formation during NSCs differentiation.

Sox4 directly represses Hes5 mRNA expression

In order to understand the mechanism by which Sox4 regulates NSC oligodendrocyte differentiation, the genes of the Notch signaling pathway were further analyzed. The Notch signaling pathway was identified in our GO-term analysis (**Figure 2G**) and is fundamental for the regulation of NSC differentiation (Ables et al., 2011; Louvi and Artavanis-Tsakonas, 2006). Amongst the genes identified, the transcription factor Hes5 was found to be induced by Sox4 in the RNA-seq data (**Figure 4A**). Moreover, we observed a binding site of Sox4 on the promoter region of *Hes5* in the ChIP-seq dataset (**Figure 4A**). Furthermore, we validated Hes5 induction by Sox4 using 2 independent shRNAs against Sox4, distinct from those utilized in the RNA-seq expreriment (**Figure 4B**). Taken together, these observations strongly suggest that Sox4 regulates Hes5 mRNA expression by binding to its promoter region. Hes5 has been shown to repress oligodendrocyte differentiation and myelination (Kondo and Raff, 2000; Liu et al., 2006). Therefore, Sox4-mediated induction of Hes5-expression may explain the inhibition of oligodendrogenesis mediated by Sox4 during NSC differentiation.

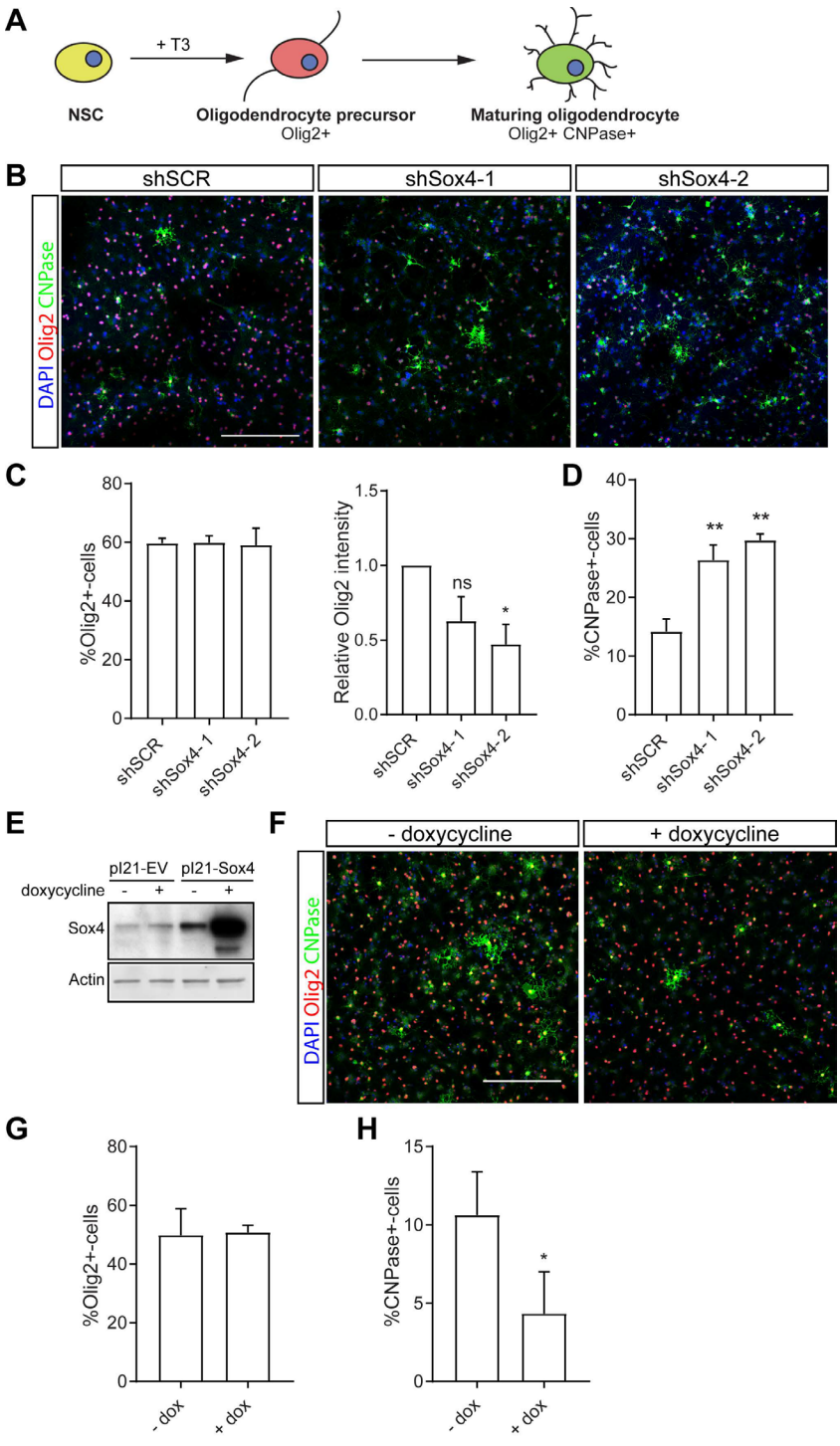
A
+ T3
NSC
Oligodendrocyte precursor
Olig2+
Maturing oligodendrocyte
Olig2+ CNPase+
B
shSCR
shSox4-1
shSox4-2
DAPI Olig2 CNPase
C
%Olig2+-cells
Relative Olig2 intensity
ns
*
D
%CNPase+-cells
**
**
E
pI21-EV
pI21-Sox4
doxycycline
Sox4
Actin
F
- doxycycline
+ doxycycline
G
%Olig2+-cells
- dox
+ dox
H
%CNPase+-cells
*

Figure 3. Sox4 represses oligodendrocyte differentiation of NSCs *in vitro*.

Sox4 KD NSCs were differentiated for 5 days in presence of T3. **(A)** Schematic description of oligodendrocyte markers used in the study. **(B)** Representative confocal images showing the expression of the oligodendrocyte markers Olig2 (red) and CNPase (green). DAPI co-stained nuclei in blue (bar=200 µm). **(C)** and **(D):** quantification of **(B)** (n=3), **=p<0.01. Sox4 OE NSCs were differentiated for 5 days in presence of T3. **(E)** Representative western blot showing inducible Sox4 overexpression in presence of doxycycline. **(F)** Representative confocal images showing the expression of the oligodendrocyte markers Olig2 (red) and CNPase (green). DAPI co-stained nuclei in blue (bar=200 µm). **(G)** and **(H):** quantification of **(F)** (n=3), *=p<0.05.

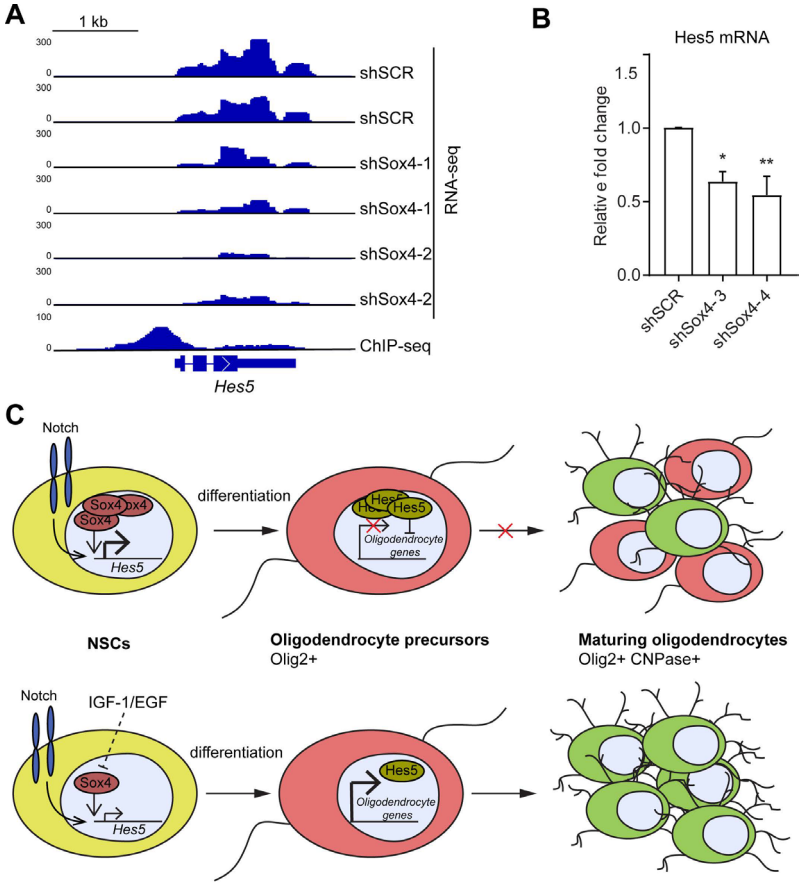

Figure 4. Sox4 induces Hes5 expression by binding to its promoter region

(A) Visualization of RNA-seq reads and Sox4 ChIP-seq profile around the genomic locus of *Hes5* in control and KD conditions. **(B)** Measure of Hes5 mRNA levels upon Sox4 KD (n=5) *=p<0.05, **=p<0.01. **(C)** Schematic model representing Sox4 inhibition of oligodendrocyte differentiation by activation of Hes5. *In vitro,* NSCs express Sox4 which induces Hes5 expression, further enhancing its induction by Notch activation. During oligodendrocyte differentiation, Hes5 represses oligodendrocyte differentiation genes, inhibiting maturation of oligodendrocyte differentiation towards Olig2+/CNPase+ cells. Upon oligodendrogenesis stimuli *in vivo*, such as IGF-1 or EGF, Sox4 levels are reduced, with consequent reduced expression of Hes5. Hence, oligodendrocyte differentiation genes are expressed, yielding a greater number of maturing oligodendrocytes.

Discussion

Here we show for the first time a role of Sox4 in regulating oligodendrocyte differentiation from murine embryonic NSCs. Sox4 modulates genes involved in CNS development, specifically repressing genes involved in oligodendrocyte differentiation, while inducing genes associated with neural and astrocyte phenotypes. Sox4 expression inhibits oligodendrocyte differentiation of NSCs *in vitro*, and Hes5, as a direct target activated by Sox4, suggesting may mediate inhibition of oligodendrocyte differentiation. These novel observations suggest that Sox4 inhibits NSC differentiation towards oligodendrocytes, possibly by inducing Hes5 expression.

Sox4, belonging to the SoxC family of transcription factors, is highly homologous with Sox11, sharing a high level of identity within the high-mobility group (HMG) domain and the C-terminal transactivation domain (TAD) (Dy et al., 2008). Several studies have provided evidence that Sox4 together with Sox11 regulate neurogenesis. Bergsland and colleagues demonstrated how during neural tube development in chicken both Sox4 and Sox11 induce the expression of pro-neural genes, βIII-tubulin and MAP2, suggesting that Sox4 and Sox11 are crucial for the later differentiation steps of neural differentiation (Bergsland et al., 2006). This is mediated by the direct induction of βIII-tubulin expression by Sox4 and Sox11. In our study we identified the genes regulated directly by Sox4 in NSCs. Interestingly, by knocking down Sox4, and without reducing with Sox11 or Sox12 expression **(Figure S1A)**, we identify multiple genes involved in CNS development. This suggests that Sox4 has an independent role from Sox11 in regulating a set of genes involved in neural development. In agreement with Bergsland and colleagues, GSEA shows that Sox4 induces pro-neural genes (Bergsland et al., 2006), supporting that Sox4 induction of a neural phenotype is conserved amongst chicken and mouse, although βIII-tubulin was not one of the Sox4-regulated genes (data not shown). This suggests that Sox4-mediated gene regulation is context dependent. Sox4 and Sox11 have been shown to promote neural progenitor proliferation and survival in mouse, as inactivation of these SoxC members induces apoptosis (Bhattaram et al., 2010). These effect can be partially explained by the activation of the expression of Tead2 by Sox4 and Sox11, since Tead2 is known to promote cell survival during organogenesis (Bhattaram et al., 2010). Sox4 and Sox11 co-expression in the adult mouse hippocampus has been shown to promote neurogenesis from neural precursor cells (Mu et al., 2012). Sox4 and Sox11 directly induce the expression of the neuronal protein doublecortin (DCX) by binding to its promoter (Mu et al., 2012). However, when compared to our datasets, we cannot find Tead2 nor DCX amongst the targets regulated by Sox4 (data not shown), suggesting once more a strong context specificity. There is evidence that Sox4 and Sox11 play *non*-redundant roles in neuronal development, since they are expressed by different neural populations during corticogenesis in mice (Chen et al., 2015). Sox11 activates the pro-neural gene NeuroD1 in early born neurons; while Sox4 is required for the maintenance of intermediated progenitors by activating Tbrain2 (Chen et al., 2015). This supports our

conclusion that Sox4 plays a unique role, independent from Sox11, in regulating NSC differentiation.

Sox4 and Sox11 have been shown to be expressed by oligodendrocyte precursors (Kuhlbrodt et al., 1998). Potzner *et al.* demonstrated that sustained expression of a Sox4 transgene induced by the myelin basic protein (MBP) promoter region, which induces Sox4 expression during oligodendrocyte differentiation, inhibiting myelination in the whole CNS (Potzner et al., 2007). Sox4 also inhibits differentiation of Schwann cell, which myelinate the axons of peripheral nerves (Bartesaghi et al., 2015). Bartesaghi and colleagues showed that transgenic expression of Sox4 in Schwann cells through the promoter of the myelin protein zero (MPZ) gene and the enhancer of MBP inhibits myelination in a mouse model of Charcot–Marie–Tooth 4C disease (Bartesaghi et al., 2015). These observations suggest that Sox4 negatively regulates oligodendrocyte differentiation and prevents myelination. Accordingly, we identify Sox4 as a negative regulator of oligodendrocyte differentiation of NSCs. Olig2 is an early marker of oligodendrocyte progenitors which is expressed by oligodendrocyte progenitors, while CNPase is a myelin associated protein that is expressed in later stages during oligodendrocyte differentiation compared to Olig2 (Miron et al., 2011). Interestingly, we observe that Sox4 does not influence the percentage of Olig2+-cells but represses the number of CNPase+-cells during NSC differentiation towards oligodendrocyte. However, a decrease in Olig2 intensity per cell was found upon Sox4 KD, although the meaning of this reduction is unclear.

Taken together, these data suggest that Sox4 does not influence the fate specification of NSCs towards the oligodendrocyte lineage, but rather their differentiation towards more mature oligodendrocytes.

Sox4 could inhibit oligodendrocyte differentiation either by inhibiting the expression of genes positively regulating this process, or inducing the expression of negative regulators (Kondo and Raff, 2000; Liu et al., 2006). The expression of Hes5, an inhibitor of oligodendrogenesis and myelination, decreases during oligodendrocyte precursor cell differentiation and overexpression of Hes5 inhibits oligodendrocyte differentiation (Kondo and Raff, 2000; Wu et al., 2003). Furthermore, Hes5$^{-/-}$ mice have shown increased myelination as shown by an increase of the myelin associated proteins proteolipid protein (PLP), ceramide galactosyl transferase (CGT), MBP, and CNPase expression (Liu et al., 2006). Specifically, Hes5 has been demonstrated to directly repress the activity of promoter regions of CGT and MBP (Liu et al., 2006). Taken together these data support the notion that Hes5 is a repressor of oligodendrocyte differentiation. We demonstrated that Sox4 directly binds to Hes5 promoter and increases its mRNA expression. The induction of Sox4 expression by Hes5 could mediate the repression of oligodendrocyte differentiation of NSCs by Sox4 **(Figure 4C)**. To validate this hypothesis it is necessary to determine whether

ectopic expression of Hes5 rescues the increase of oligodendrocyte differentiation mediated by Sox4. Hes5 has been described, together with Hes1, to promote NSC maintenance during embryogenesis in mice (Ohtsuka et al., 2001). It is possible that Sox4, through induction of Hes5 expression during oligodendrogenesis, induces maintenance of neural progenitor characteristics. Therefore it would be relevant to measure the levels of NSC markers, such as Nestin or Sox2 during oligodendrocyte differentiation upon Sox4 depletion (De Filippis and Binda, 2012). Since Hes5 is a downstream effector of Notch, it would also be important to investigate whether Notch activation induces Sox4 activation in NSCs, as a way to further enhance Hes5 expression. Moreover it would be relevant to investigate whether oligodendrogenesis stimuli, such as epidermal growth factor (EGF) and insuling growth factor-1 (IGF-1), could reduce Sox4 levels to promote oligodendrocyte differentiation (Patel and Klein, 2011). In contrast to our findings, Kuwajima *et al.* recently provided preliminary data suggested that Sox4 directly represses Hes5 expression in retinal ganglion cells (RGC) in mice. However, this data needs to be validated in relevant cell types since the only evidence for Hes5 repression was provided by utilizing a *Hes5* promoter-luciferase assay in HEK293 cells in combination with Sox4 overexpression (Kuwajima et al., 2017). However, if correct, this finding would be another example of the context-dependent role of Sox4. Interestingly, a member of the SoxB family, Sox21, has been described to promote hippocampal neurogenesis in adult mice by repressing Hes5 expression (Matsuda et al., 2012). It would be relevant to investigate whether Sox21-mediated repression of Hes5 expression promotes oligodendrogenesis, in contrast to Sox4. In conclusion these observations suggest that Sox4 represses NSC differentiation towards oligodendrocytes by inducing the expression of Hes5, shedding light on a novel regulator of oligodendrocyte differentiation.

Methods

qPCR

RNA was isolated using the RNAeasy kit (Qiagen, Hiden, Germany) according to manufacturers' instruction. Reverse transcriptase reaction for the generation of cDNA was performed using iScript cDNA synthesis kit (Bio-rad, Hercules, CA) followed by real-time quantification with the LightCycler 96 Real-Time PCR System (Roche Life Sciences, Penzberg, Germany) using SYBR Green Supermix (Bio-Rad) for cDNA application following manufacturers' protocol. Relative expression was calculated with the ΔΔCt method using hypoxanthine-guanine phosphoribosyltransferase (Hprt) to normalize.

List of qPCR primers used

Primer	Sequence
FW-Sox4	CCAGTTCTTGCACGCTGTTT
REV-Sox4	TGTTGCAAGGTAGGAAGCCA
FW-Hes5	GAGAAAAACCGACTGCGGAAG
REV-Hes5	GCGAAGGCTTTGCTGTGTTT
FW-Hprt	CCTAAGATGAGCGCAAGTTGAA
REV-Hprt	CCACAGGACTAGAACACCTGCTAA

FW: Forward primer

REV: Reverse primer

Cell culture

NSCs derived from embryonic day 14.5 CD-1 mice were purchased from R&D Systems, Minneapolis, MN. NSCs were grown as neurospheres in DMEM:F12 medium (Thermo Scientific, Waltham, MA) supplemented with 50x B-27 (Thermo scientific) with 20 ng/mL EGF and 20 ng/mL bFGF (both Peprotech, Rocky Hill, NJ) plus antibiotics (Thermo Scientific). For experiments NSCs were cultured on 35 mm glasses coated with poli-L-ornithine (10µ/mL, Sigma Aldrich) and Laminin (1µ/mL, Sigma Aldrich) and let adhere for 48h. For the differentiation assay, NSC differentiation was induced by growth factor withdrawal and addition of 50ng/mL of triiodothyronine (T3) (Sigma Aldrich). Cells were fixed 5 days after in formaldehyde (FA) 4%. Lentiviral particles were generated as previously described (Vervoort et al., 2013a). NSCs were transduced twice in adherence on plates coated with poli-L-ornithine (10µ/mL) Sigma Aldrich) and Laminin (5µ/mL, Sigma Aldrich). The following shRNAs vectors were used: shSox4-1 (TRCN0000012078), shSox4-2 (TRCN0000012080), shSox4-3 (TRCN0000234110), shSox4-4 (TRCN0000012081) and shSCR (SHC002), all from Sigma Aldrich. The cells were selected with puromycin (0.5 µg/mL). For the overexpression experiment the coding region of murine Sox4 was amplified from pcDNA3.1-FLAG-Sox4 (Beekman JM *et al,* 2012(Hu et al., 2006) with the addition of an N-terminal hemagglutinin (HA) tag peptide, cloned into pDONR221 (Thermo Fisher Scientific) with Gateway BP Clonase II Enzyme mix (Thermo Fisher Scientific) and then into the lentiviral vector pINDUCER21 (ORF-EG) (from Stephen Elledge & Thomas Westbrook) (Addgene plasmid # 46948) (Yang et al., 2011) with Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific) according to manufacturer instructions. Overexpression was induced with the addition of 0.5 µg/mL of doxycycline (Sigma-Aldrich) to the culture medium.

Immunofluorescence

For differentiation experiments, cells were fixed in 4% FA and stained with rabbit anti-Olig2 (Millipore, Bedford, MA), mouse anti-CNPase (Abcam, Cambridge, UK), followed by incubation with donkey anti-rabbit AlexaFluor-555 or goat anti-mouse AlexaFluor-488 (both Thermo Scientifics, 1:250). Nuclei were counterstained with 4',6-Diamidino-2-Phenylindole (DAPI). Fluorescent confocal images were captured with an AxioCam MRm (Carl Zeiss, Sliedrecht, The Netherlands) on a LSM 700 confocal microscope (Carl Zeiss). The number of Olig2- and CNPase-positive cells were quantified by counting cells in 8 different fields of the culture dish.

Western blot

Cells were lysed directly in the plate with Laemmli buffer (0.12 mol/L Tris-HCL (pH6.8), 4% SDS and 20% glycerol). Protein concentration was measured with the Lowry assay. 40 µg of each sample were analyzed by sodium dodecil sulfate polyacrilammide gel (SDS-PAGE) and transferred by electrophoresis onto polyvinylidene difluoride membrane (Millipore). The membranes were blocked using 5% milk protein in TBST (0.3% Tween, 10 mM Tris pH 8 and 150 mM NaCl in H_2O) and probed with anti-Sox4 (Diagenode, Liège, Belgium, CS-129-100, 1:3000) and anti-Tubulin (Sigma Aldrich, St. Louis, MS, T5168, 1:50000). Signal was detected using Amersham ECL Western Blotting Detection Reagent (Little Chalfont, United Kingdom).

Chromatin Immunoprecipitation and sequencing

Before the Chromatin Immunoprecipitation (ChIP) and sequencing, NSCs were grown as neurospheres in complete medium in 6-wells. ChIP was performed as previously described (van Boxtel et al., 2013). Briefly, crosslink was performed with disuccinimidyl glutarate (DSG) (Thermo Scientifis) for 45 minutes followed by 30 minutes incubation with formaldehyde 1%. The reaction was blocked with glycine 100 mM. Shearing was performed using Covaris S2 (Covaris, Woburn, MA) for 8 min at maximum intensity. The sonicated chromatin was incubated O/N at 4°C in presence of 10 µg of rabbit anti-Sox4 (Diagenode, CS-129-100) coupled to A/G sepharose beads (Santa Cruz Biotechnology). Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, MA) was used for End-repair, A-tailing and ligation of sequence adaptors. Samples were amplified by PCR and the libraries were size-selected in the 200-500 bp range. Bar-coded libraries were sequenced on Illumina NextSeq500 sequencer as previously described (Peeters et al., 2015) (50bp, single-end, Utrecht sequencing facility, Utrecht Medical Center, Utrecht, The Netherlands).

RNA sequencing

Total RNA was extracted from adherent NSCs cultured on 6-well coated plastic plates for 48h using the RNAeasy Kit (Qiagen). RNA quality was tested on the Bioanalyzer (Agilent, Santa Clara, CA) and sample quality was optimal with RNA integrity number (RIN) higher than 9.0. Sample preparation was performed using Poly(A)Purist MAG Kit (Thermo Scientific)

according to manufacturers' instructions. Isolated mRNA was subsequently repurified using mRNA-ONLY Eukaryotic mRNA Isolation Kit (Epicentre (Illumina, Inc.), Madison, WI, USA). Sequencing libraries were prepared using SOLiD Total RNA-Seq Kit (Applied Biosystems Life Technologies) according to the standard protocol recommendations and sequenced on SOLiD Wildfire sequencer to produce 50bp reads as previously described (van Boxtel et al., 2013).

NGS data analysis

For RNA-sequencing analysis sequencing reads were mapped against the reference genome (mm10 assembly) using TopHat v2.0.9 as previously described (Trapnell et al., 2012). Only uniquely mapped reads were selected for further analysis. In brief, guide transcripts were assembled using CuffLinks v2.2.1. Reads were quartile normalized using the –library-norm-method quartile option. Differential gene analysis was performed using CuffDiff. Cluster 3.0 and Java TreeView software v1.1.6 were used for visualization of heatmaps. ChIP-sequencing reads were mapped with Bowtie 2.1.0 against the reference genome (mm10) using default settings (Langmead and Salzberg, 2012). Peaks were called with MACS2 software using the input as a control (Heinz et al., 2010). Mapped fragments were extended according to the average fragment size and converted to TDF files, visualized with IGV tools v2.3.36 and represented as coverage normalized tracks (Robinson et al., 2011). SAMtools was used for manipulation of SAM and BAM files; manipulation of BED file format was performed with BEDtools (Li et al., 2009; Quinlan and Hall, 2010). Motif discovery, peak annotation and generation of histograms were performed using HOMER software. For motif discovery the 200 bp sequence surrounding each peak was examined and motifs with length of 8, 10 and 12 nt were searched. Motifs were identified through *de novo* motif discovery. For functional annotation and Sox4 target identification the software GREAT was used, where genes that presented peaks within 5kb from the TSS were selected (McLean et al., 2010). Average distances to TSS of peaks were calculated with GREAT. The ToppGene suite database (https://toppgene.cchmc.org/enrichment.jsp) and the REVIGO visualization tool were used for GO-term analysis of differentially expressed and Sox4-bound genes (Chen et al., 2009; Supek et al., 2011).

Gene Set Enrichment Analysis

For Gene Set Enrichment Analysis (GSEA) pre-ranked analysis was performed with the GSEA software probing for enrichment of curated gene sets (C2) in the RNA-seq dataset ranked by log fold-change (Carbon et al., 2009; Subramanian et al., 2005).

Statistics

Data are expressed as mean ± SEM and regarded statistically significant if $p < 0.05$. Data were analyzed using one-way ANOVA with Dunnett's post-test. For the differentiation experiment with Sox4 overexpression data were analyzed using two-tailed paired Student's t-test.

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Supplementary figures

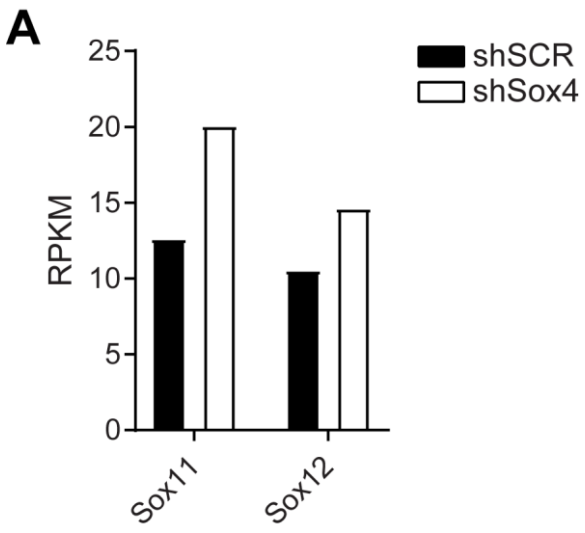

Figure S1. Sox4 KD in NSCs does not reduce Sox11 and Sox12 expression

(A) Quantification of expression levels of Sox11 and Sox12 upon Sox4 KD in the RNA-seq dataset.

CHAPTER 4

Delayed administration of neural stem cells after hypoxia-ischemia reduces sensorimotor deficits, cerebral lesion size and neuroinflammation in neonatal mice.

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Abstract

Background

Hypoxic-ischemic (HI) encephalopathy causes mortality and severe morbidity in neonates. Treatments with a therapeutic window >6 hours are currently not available. Here we explored whether delayed transplantation of allogenic neural stem cells (NSCs) at 10 days after HI could be a tool to repair HI brain injury and improve behavioral impairments.

Methods

HI was induced in 9 day-old mice. Animals received NSCs or vehicle intracranially in the hippocampus at 10 days post-HI. Sensorimotor performance was assessed by cylinder rearing test. Lesion size, synaptic integrity and fate of injected NSCs were determined by immuno-stainings. Neuroinflammation was studied by immuno-stainings of brain sections, primary glial cultures and TNFα ELISA.

Results

NSC transplantation at 10 days post-insult induced long-term improvement of motor performance and synaptic integrity, and reduced lesion size compared to vehicle-treatment. HI-induced neuroinflammation was reduced after NSC treatment, at least partially by factors secreted by NSCs. Injected NSCs migrated towards and localized at the damaged hippocampus. Transplanted NSCs differentiated towards the neuronal lineage and formed a niche with endogenous precursors.

Conclusion

Our study provides evidence of the efficacy of NSC transplantation late after HI as a tool to reduce neonatal HI brain injury through regeneration of the lesion.

Introduction

Neural stem cells (NSCs) are pluripotent cells that can self-renew and differentiate into neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992). NSCs can be found both in the developing embryo during neurogenesis identified as radial glia, and in specialized niches of the adult brain such as the subventricular zone (SVZ) and the granular layer of the dentate gyrus (DG) of the hippocampus (Palmer et al., 2000). NSCs can be isolated from the developing cortex or from the adult niches and expanded in vitro as neurospheres (Giachino et al., 2009). Several studies using animal models of neurodegenerative pathologies show that NSC transplantation is a promising tool for treating conditions affecting the central nervous system (Martino and Pluchino, 2006). NSC transplantation has been shown to improve cognition in a transgenic mouse model of Alzheimer's disease (Blurton-Jones et al., 2009), to induce recovery in a murine model of multiple sclerosis (Pluchino et al., 2003), to improve motor function in mice with Machado-Joseph disease (Mendonca et al., 2015), and to promote recovery after neonatal excitotoxic brain injury (Titomanlio et al., 2011).

Hypoxia-ischemia (HI) during the perinatal period is a severe condition with high mortality and major long-term neurodevelopmental consequences for the survivors (de Haan et al., 2006; Ferriero, 2004). Affected neonates often suffer from brain damage as a major consequence of HI leading to long-term sequelae including cerebral palsy, mental retardation, seizures and, behavioral and psychological problems.

After a HI insult, NSCs expand within the subventricular zone (SVZ) to participate in an endogenous regenerative response (Buono et al., 2015) (Felling et al., 2006). However, this endogenous restorative capacity is limited due to HI-induced apoptosis of NSC-derived neural precursors hampering the generation of new functional neurons and glial cells (Brazel et al., 2004). To circumvent these limitations and improve the endogenous regenerative potential of the neonatal brain after HI, a number of preclinical studies by our group and others aimed at either replacing the endogenous stem cells or boost their differentiation capacity, for instance by intracranial delivery of NSCs or intranasal application of mesenchymal stem cells (Daadi et al., 2010; Donega et al., 2013; van Velthoven et al., 2010).

Current treatment options for neonatal HI brain injury are scarce and are mostly aimed at reducing early brain damage (e.g. by whole body hypothermia) and therefore rely on a strictly timed application within a few hours (<6 hours) after the insult (Azzopardi et al., 2009; Gluckman et al., 2005). Therefore there is a need for new efficacious regenerative strategies like stem cell transplantation that can be applied with a wider therapeutic time-window than the established interventions at present.

A few recent pre-clinical studies have shown how NSC transplantation ameliorates functional impairment and reduces tissue damage after neonatal HI. However, these studies only reported administration of NSCs within 1-3 days after HI (Ashwal et al., 2014; Daadi et al., 2010; Rosenblum et al., 2015). The aim of the present study is to investigate whether late administration of NSCs (at 10 days after insult) has a beneficial effect on HI-induced deficits in motor behavior, lesion size and neuroinflammation. We also aimed to elucidate the engraftment and fate of transplanted NSCs.

Results

Intracranial NSC treatment at 10 days after HI improves motor performance

P9 mouse pups underwent HI and were treated with an intracranial injection of 100.000 allogenic NSCs or vehicle solution at 10 days post-insult (for experimental set-up see Figure 1, panel a). By using the cylinder rearing test (CRT) we investigated whether delayed NSCs transplantation could restore deficits in motor behavior induced by HI. At 10, 28 and 56 days after the HI insult, HI-VEH animals showed a significant preference to use the non-impaired forepaw when compared to sham-operated littermates (SHAM) indicating HI-induced motor impairment (Figure 1, panel b). NSC-treated animals showed a strong reduction in non-impaired forepaw preference at both 28 and 56 days post-HI

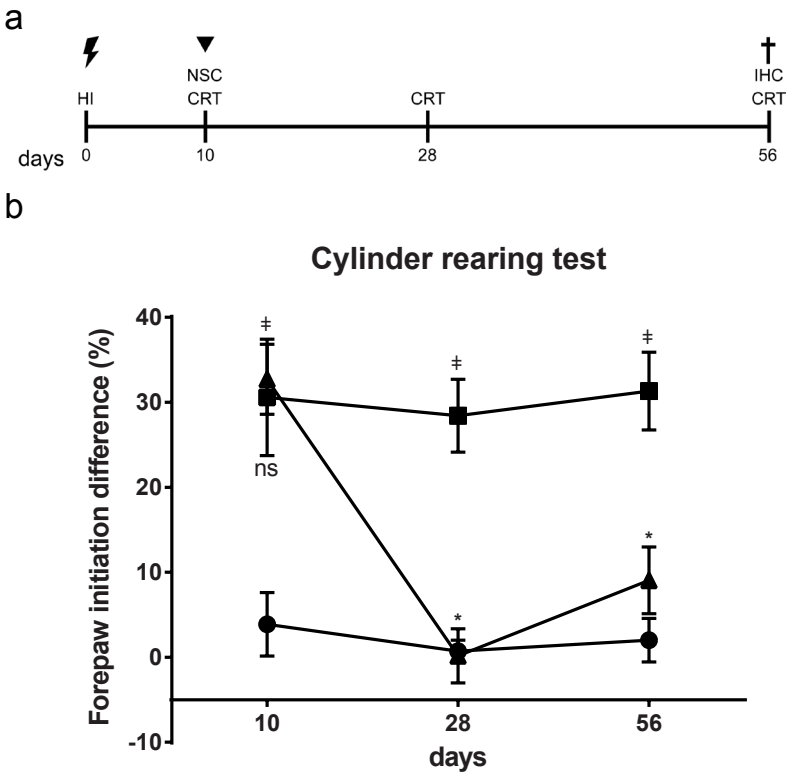


Figure 1: Effect of NSC treatment on sensorimotor behavior over time.

(a) Experimental set-up showing day of HI surgery or sham operation (lightning), intracranial NSC or vehicle treatment (arrowhead) and termination at day 56 after HI (cross). Timepoints of CRT testing are shown over time (i.e. 10, 28 and 56 days after HI) **(b)** CRT results show that NSC treatment improves motor performance at 28 and 56 days after HI as compared to vehicle treatment. CRT at 10 days post-HI is performed prior to treatment. Circles: SHAM; squares: VEH; triangles: NSC. Animal numbers: 10 days SHAM: n= 8, VEH: n=7, NSC: n=9. 28 days SHAM: n= 9, VEH: n=9, NSC: n=7. 56 days SHAM: n= 9, VEH: n=6, NSC: n=9. ≠ p<0.001: VEH vs sham-controls; * p<0.001: NSC vs VEH; ns = not significant with two-way ANOVA and LSD post-test.

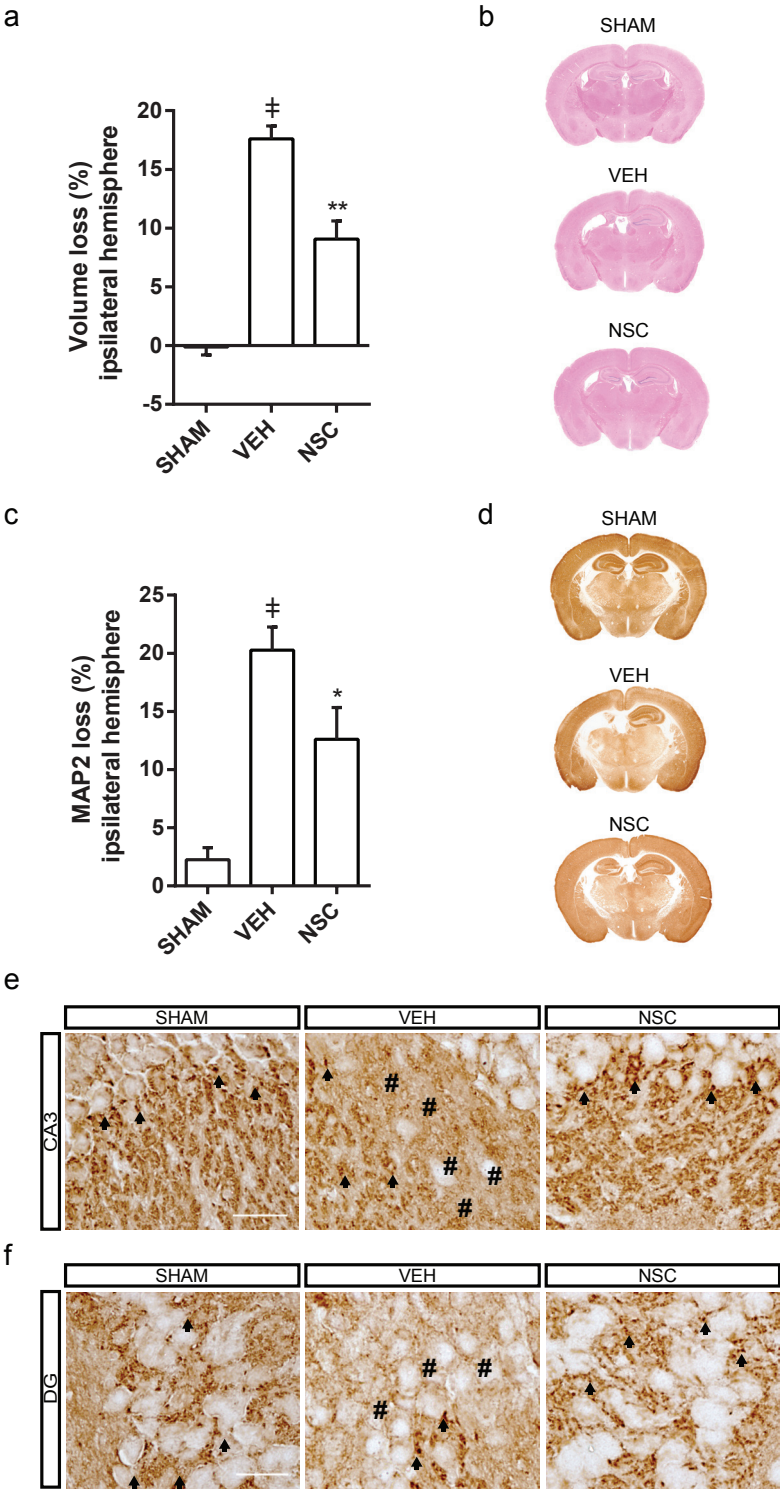
when compared to HI-VEH mice (Figure 1, panel b). The reduction in motor impairment after NSC treatment remained steady between 28 and 56 days after the insult indicating long-term improvement of motor behavior. In order to assure that the HI-VEH and HI-NSC experimental groups had a comparable degree of motor impairment upon HI, mice were tested in the CRT prior to treatment (10 days after HI) before randomly dividing the pups among the different groups. Figure 1, panel b shows that HI-induced preference to use the non-impaired forepaw was similar in VEH- and NSC-treated mouse pups prior to treatment (i.e. day 10).

Intracranial NSC treatment reduces infarct size and preserves synaptic integrity in the ipsilateral hemisphere

To assess the long-term effect of NSC treatment on lesion size and specific neuronal and white matter damage, mice were sacrificed at 56 days post-HI (Figure 1, panel a). Brains were stained for hematoxylin eosin (HE) to assess ipsilateral volume loss, for microtubule-associated protein 2 (MAP2) to assess ipsilateral neuronal loss, for synaptophysin to assess synaptic integrity. Analysis of HE staining showed that HI induced 18 ± 3% loss of ipsilateral area in VEH-treated animals with a clear lesion in the hippocampal area and some loss of volume in the cortex and thalamic regions (Figure 2, panel a,b). Importantly, NSC treatment significantly reduced volume loss to only 9 ± 4% loss in the ipsilateral hemisphere when compared to VEH (Figure 2, panel a,b). Sham-operated littermates did not show any area loss.

In addition, Figure 2, panel c shows that HI-induced ipsilateral neuronal damage was significantly reduced after NSC treatment (13 ± 8% MAP2 loss) when compared to VEH-treated animals (20 ± 6% MAP2 loss). No MAP2 loss was observed in sham-operated animals (Figure 2, panel c,d). MAP2 loss in HI animals was primarily observed in the hippocampal area.

As is shown in Figure 2, panel e, f, synaptophysin-positive vesicles (dark-brown dots, see arrows) were observed throughout the hippocampus in sham-operated animals (arrows), indicating intact functional neurons. HI induced massive loss of synaptophysin staining both in the CA3 and DG areas of the hippocampus respectively (number signs) (Figure 2, panel e, f). In some vehicle-treated animals the hippocampus was severely infarcted with complete loss of synaptophysin staining (data not shown). NSC-treated animals showed the presence of synaptophysin-positive vesicles (arrows) in the CA3 and DG. These data indicate that upon HI, NSCs might prevent the loss of previously formed synapses or might be involved in formation of new synapses during repair of the damaged areas (Figure 2, panel e, f).


a
Volume loss (%)
ipsilateral hemisphere
20
15
10
5
0
-5
‡
**
SHAM
VEH
NSC
b
SHAM
VEH
NSC
c
MAP2 loss (%)
ipsilateral hemisphere
25
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5
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SHAM
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d
SHAM
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e
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CA3
f
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VEH
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Figure 2: Effect of NSC treatment on lesion volume, gray matter injury and synapse loss at 56 days after HI.

(a) NSC treatment significantly reduces HI-induced volume loss in the ipsilateral hemisphere. **(b)** Representative sections of HE staining in all experimental groups**.** (**c**) NSC treatment significantly reduced HI-induced MAP2 loss in the ipsilateral hemisphere. **(d)** Representative sections of MAP2 staining in all experimental groups. **(e-f)** Representative sections of synaptophysin staining at CA3 **(e)** and DG **(d)** areas of the hippocampus. Synaptophysin-positive vesicles are shown at arrowheads. NSC treatment reduces loss of synaptophysin staining as observed in vehicle-treated littermates (number signs). Scale bar 30 µm. ≠ $p<0.001$: VEH versus sham-control; * $p<0.05$, ** $p<0.001$: NSC vs VEH with one-way ANOVA and LSD post-test. Animal numbers: HE SHAM: n= 9, VEH: n=9, NSC: n=9. MAP2 SHAM: n= 9, VEH: n=9, NSC: n=9.

Localization and fate of transplanted NSCs

In order to investigate where NSCs home to in the HI-damaged brain after intracranial transplantation, NSC were labeled with PKH26 and injected intracranially into mice at 10 days post-HI (for experimental set-up see Figure 3, panel a). Animals were sacrificed 3 days after transplantation and brains were sectioned at the hippocampal level as the injection site was within the damaged ipsilateral hippocampus (number sign Figure 3, panel b). At 3 days after injection, PKH26 signal was detectable in (1) multiple areas of the damaged ipsilateral hippocampus aligning the ipsilateral dentate gyrus (DG) (red boxes Figure 3, panel b), (2) the area of the dorsal third ventricle (blue boxes Figure 3, panel b) and (3) the third ventricle (green box Figure 3, panel b).

In order to determine the fate of the PKH26+ NSCs, immunofluorescent analyses for progenitor- and lineage-specific markers were performed. At 3 days after transplantation $PKH26^+$ cells are not expressing the stem cell marker Nestin (Figure 3, panel c, d). However $PHK26^+$ cells were found in proximity of $Nestin^+$ $PHK26^-$ cells (Figure 3, panel c, d) suggesting that the injected exogenous NSCs might interact with endogenous progenitors in the brain as previously reported (Martino and Pluchino, 2006). Staining for the neuroblast marker Doublecortin (DCX) revealed the presence of double-positive $PKH26^+/DCX^+$ cells (Figure 3, panel c, d) indicating that at least a part of the injected NSCs had differentiated towards young migrating neuroblasts at 3 days post-injection. Conversely, $GFAP^+$ astrocytes could also be found surrounding the areas of PKH26 signal, but no cellular co-localization of GFAP and PKH26 signal could be identified (Figure 3, panel c, d). These data indicate that the injected NSCs are not differentiating towards the astrocyte lineage.

Migration of NSCs towards the lesion site after transplantation in the contralateral hippocampus

In order to investigate the migratory capacity of transplanted NSCs towards the damaged areas, we injected NSCs in the contralateral, undamaged hippocampus (Figure 4). At 5 days after transplantation the NSCs were detected within the DG of the ipsilateral hippocampus and in other hippocampal regions surrounding the lesion (Figure 4). Strikingly, hardly any NSCs were detected in the contralateral hippocampus at the injection site, meaning that the majority of the transplanted cells migrated after transplantation, most likely through a chemoattractive gradient generated by the damaged milieu.

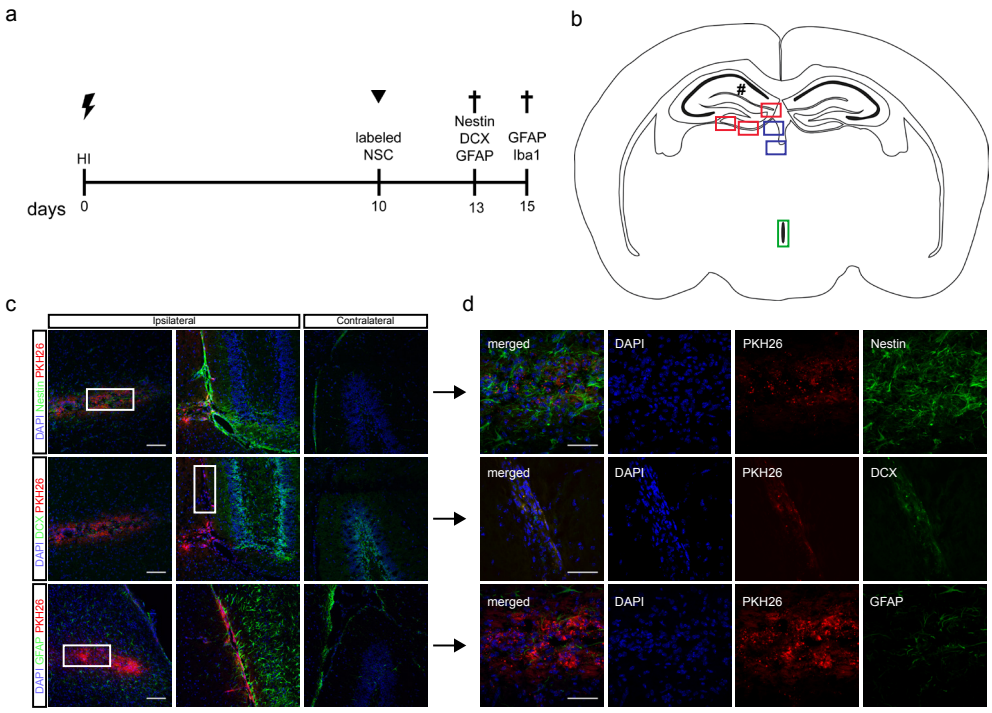

Figure 3: Localization and fate of injected NSCs in the HI-damaged brain.

(a) Experimental set-up showing day of HI surgery or sham operation (lightning), intracranial PKH-labeled NSC or vehicle treatment (arrowhead) and termination days (crosses; i.e. 13 and 15 days post-HI). **(b)** Coronal brain section at hippocampal level illustrating the injection site of NSCs (number sign) and showing the localization of PKH26 signal at the hippocampus (red boxes), dorsal third ventricle (blue boxes) and third ventricle (green box). (**c**) Representative sections of mice sacrificed 3 days after NSC treatment showing localization of PHK26 (red) signal at the ipsilateral hippocampus. Sections were stained for Nestin, DCX and GFAP (green) as indicated and nuclei counterstained with DAPI (blue). No PKH26 signal was detected in the contralateral hippocampus. Boxes indicate areas depicted in Figure 3, panel d. Scale bar 100 µm. **(d)** Z-stack images showing co-localization (yellow) of PKH26 signal (red) with Nestin, DCX and GFAP (green) as indicated. Nuclei were counterstained with DAPI (blue). Scale bar 50 µm.

Neuroinflammation in the HI-damaged brain is attenuated by NSC transplantation

We next determined whether NSC treatment could have an effect on the neuroinflammatory response as a consequence of the HI insult. We determined Iba-1 and GFAP expression at 5 days after transplantation (15 days after HI) as measures of microglia activation and astrogliosis respectively (see Figure 3, panel a for set-up). Figure 5, panel a-d shows that HI induces a strong upregulation of Iba-1 and GFAP at 15 days after HI in vehicle-treated animals, especially in the hippocampal area. NSC treatment reduced the upregulation of the Iba-1+ and GFAP+ signal in this area almost completely at 5 days after transplantation (Figure, panel 5a-d) indicating that NSC transplantation dampens the neuroinflammatory response. In order to investigate whether the observed dampening of neuroinflammation could be directly mediated by the NSCs secretome, primary microglia were cultured in presence of conditioned medium (CM) derived from cultured NSCs or control non-conditioned medium

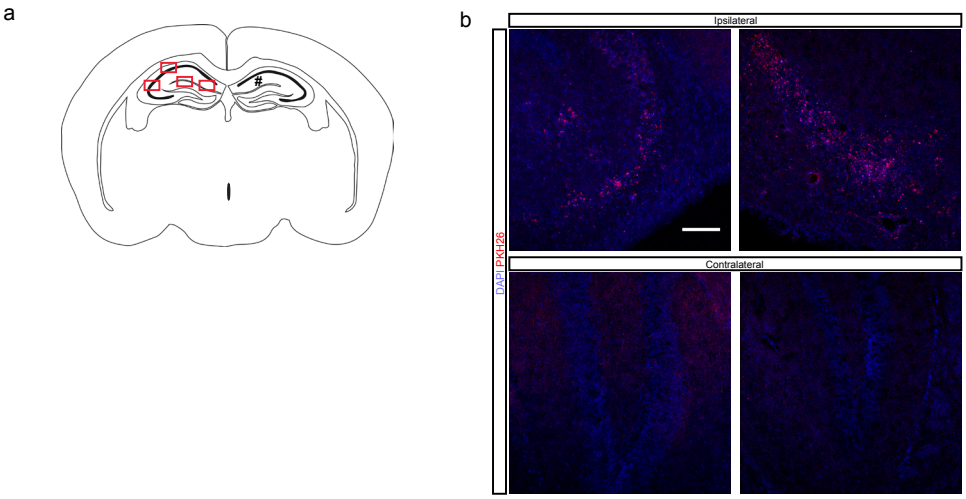

Figure 4: NSCs migrate to the lesion site.

(a) Coronal brain section at hippocampal level illustrating the injection site of NSCs (number sign) in the contralateral hippocampus and showing the localization of PKH26 signal in the ipsilateral hippocampus (red boxes). **(b)** Representative pictures of mice sacrificed 5 days after NSC treatment showing localization of PHK26 (red) signal in the ipsilateral hippocampus. Hardly any PKH26 signal was detected in the contralateral hippocampus. Nuclei were counterstained with DAPI (blue). Scale bar 100 µm.

(CTR). Figure 5, panel e shows that microglia cultured in CM produced significantly less TNFα, when compared to microglia cultured in CTR medium. Moreover, Iba1 expression level per cell was markedly decreased after CM culture when compared to CTR (Figure 5, panel f,g). As both TNFα production and Iba1 expression levels are markers of microglial activation (Ito et al., 2001; Kuno et al., 2005), these data together indicate that reduced microglial activation can be a direct effect of the NSCs' secretome.

Discussion

The present study shows the beneficial effect of late allogenic intracranial NSC transplantation for HI brain injury. We show that NSCs are remarkably potent in attenuating motor impairment in HI-injured mice with a stable and long-lasting effect. Moreover, the HI-induced ipsilateral volume loss and neuronal loss was reduced after late NSC treatment. We have shown before that the HI-induced lesion in our model is already fully established at 3 days post-HI showing disintegration of the hippocampal architecture (Bonestroo et al., 2015). This previous study showed that between day 3 and 15 after HI there was no significant increase in lesion size indicating that the applied NSCs at day 10 after HI actually repaired the established lesion. Although ipsilateral hippocampal volume was not recovered fully, MAP2 staining showed that NSCs aided in repair and reconstruction of the hippocampal structure. Synaptic integrity in the ipsilateral hippocampus was maintained or repaired after transplantation of NSCs. We observed that transplanted NSCs had differentiated into DCX+ neuroblasts. These neuroblasts were found in proximity of

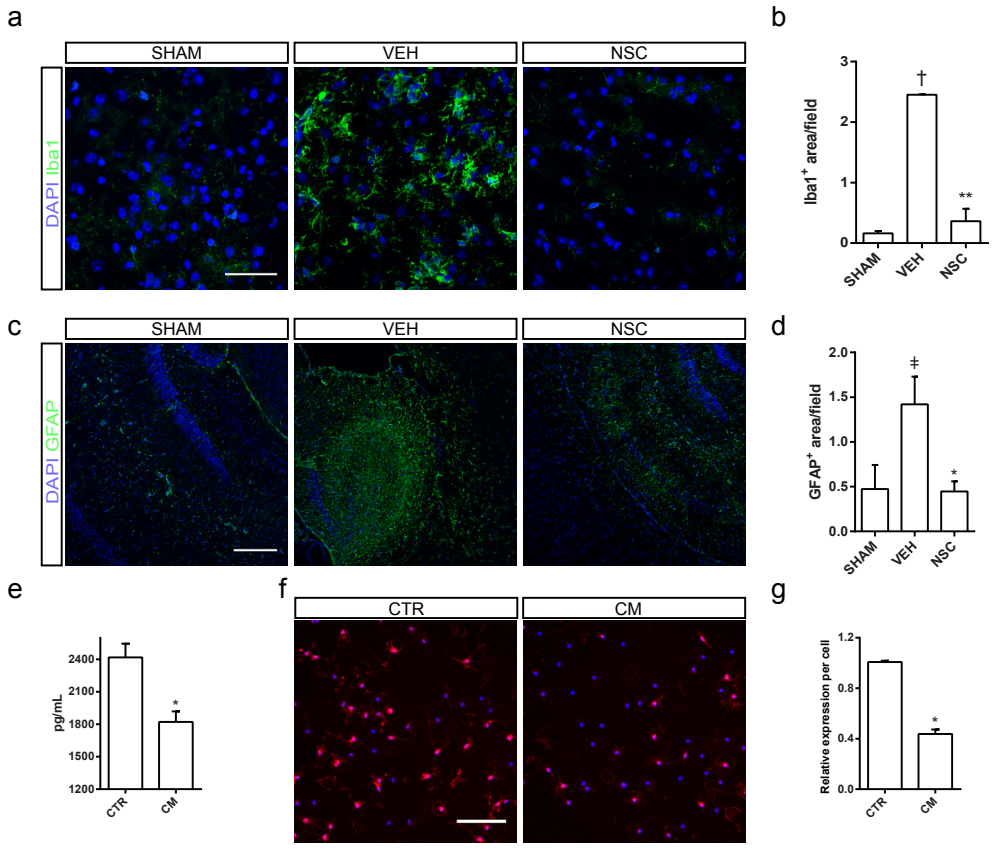

Figure 5: Effect of NSC treatment on neuroinflammation after HI.

For experimental set-up see Figure 3, panel a. **(a)** Representative sections showing microglia activation (Iba1 (green)) in the ipsilateral hippocampus. Scale bar 50 µm. **(b)** Quantification of Iba-1-positive signal showing that NSC treatment significantly reduces microglia activation. **(c)** Representative sections showing GFAP (green) signal in the ipsilateral hippocampus. Scale bar 250 µm. **(d)** Quantification of GFAP-positive signal showing that NSC treatment significantly reduces astrocyte activation ≠ p<0.05, † p<0.005: HI-vehicle vs sham-control; * <0.05, ** p<0.001: NSC treatment vs vehicle treatment analyzed with one-way ANOVA, LSD post-test. Animal numbers: Iba1: SHAM: n=2, VEH: n=3, NSC: n=5. GFAP SHAM: n=2, VEH: n=3, NSC: n=5. **(e)** TNFα levels secreted by primary cultured microglia are reduced after incubation with NSC-conditioned medium (CM) vs control medium treatment (CTR). * p<0.01. **(f)** Representative pictures showing Iba1 staining of primary microglia cultured in CTR or CM. Scale bar 100 µm. **(g)** Quantification of Iba1-positive signal per cell in CM vs CTR condition. * p<0.01. **e+g:** CTR vs CM two-tailed, unpaired Student's t-test.

endogenous astrocytes and progenitor cells indicating formation of a neurogenic niche in the lesion area. Interestingly we showed that transplanted NSCs have the capacity to migrate to the damaged area when injected at a site more distant from the lesion. Additionally, the neuroinflammatory reaction evoked by HI, as measured by microglia activation and gliosis in the area of the ipsilateral hippocampus, were strongly reduced by NSC treatment. Reduction of microglia activation could be directly mediated by the NSCs secretome, as we observed a reduction in TNFα production and Iba1 expression by microglia when cultured in presence of NSC-conditioned medium.

There are a few previous studies that have explored the therapeutic potential of NSC transplantation in different ischemic models. Daadi et al. investigated the effect of intracranial human NSC transplantation at 24 hours after HI in 7 day old rats(Daadi et al., 2010). Consistent with our results, Daadi et al. showed an improvement in motor outcome in NSC-treated animals upon HI. Moreover, this study showed that transplanted human NSCs differentiated towards the neuronal as well as the astrocytic lineage (NSCs becoming GFAP-positive cells). These data are partially in contrast with our results as we showed that all the detected NSCs had differentiated towards young migrating neuroblasts (DCX-positive cells) and we did not detect any NSCs expressing the astrocyte marker GFAP. This could be explained by the difference in the timing of transplantation between our study and the one from Daadi and his colleagues. In line with this explanation, Rosenblum et al. reported how early (6-24 hours) transplantation of NSCs in adult stroke mice led to increased differentiation of NSCs into astrocytes, however delayed (7-14 days) transplantation resulted in neuronal differentiation (Rosenblum et al., 2012). Moreover, Titomanlio et al. show that NSCs implanted into the neonatal excitotoxic injured brain early after damage (4-72 hours) differentiate towards both neurons and oligodendrocytes, before undergoing apoptosis (Titomanlio et al., 2011). These studies together indicate that the milieu of the damaged brain might change over time modulating the differentiation and survival capacity of the grafted NSCs and that it could be more beneficial to delay regenerative treatment of patients with HIE in order to improve selective neuronal repair.

Daadi et al. did not observe any effect on lesion size after NSC treatment whilst we observed a potent beneficial effect of late NSC treatment on gray matter loss. This difference could be explained by the source of the transplanted NSCs, as murine (although allogenic) NSCs used in our present study might engraft better than human NSCs in the murine host brain. This hypothesis could be supported by the fact that Daadi et al. observed an increase in Iba1-positive microglia in the transplanted area, whereas our data showed a reduction of Iba1-positive cells in the area surrounding the damaged hippocampus, indicating that the xenograft of human NSCs might elicit an inflammatory response in the brain.

Another study using neonatal HI mice showed the interaction of the host brain with mouse NSC grafts seeded onto a polymer scaffold, which was implanted at 7 days after HI in the infarction cavity (Park et al., 2002). This study showed how the grafted NSCs differentiated into neurons and oligodendrocytes, resulting in long-distance neuronal projections. Consistent with our study, Park and colleagues showed that the neuroinflammatory response, i.e. astroglial scar formation and microglia infiltration, were minimized after NSC transplantation.

Previous studies have shown that transplanted NSCs do express mature neuronal markers within several days after transplantation (Daadi et al., 2010; Park et al., 2002). Although

we did not assess long-term fate of the transplanted NSCs, our histological and behavioral data indicate that NSC transplantation eventually led to long-term functional recovery and regeneration of the hippocampal structure. We suggest therefore that the transplanted NSCs indeed might have integrated as functional differentiated neurons. In line, our data on synaptic integrity showed a potent effect of NSC treatment on synapse formation in the hippocampal area. Increased synaptophysin expression after NSC treatment can be a result of preservation of existing synapses or induction of new synapses by newly-formed neurons from the transplant.

The transplanted NSCs were detected in damaged areas of the hippocampus when transplanted in the *contralateral* hemisphere, indicating that NSCs are capable of migrating towards damage signals. This is in line with previous studies showing that injected NSCs can respond to chemoattractants (like stromal cell-derived factor 1 alpha (SDF-1alpha)) and migrate towards injury sites (Imitola et al., 2004). This finding is in favor of a possible clinical application of NSC transplantation as less invasive administration routes (e.g. intravenous, intranasal) might result in NSCs successfully reaching the lesion site.

Besides possible integration of the NSCs to replace damaged tissue, the beneficial effects we observe in HI mice after NSC treatment can also be explained by indirect secretory effects of the grafted NSCs. The inhibition of microglial activation by the NSCs secretome as shown in our study indicates that NSCs secrete anti-inflammatory factors that dampen the inflammation at the injury site thereby preventing aggravation of HI brain damage.

In addition, NSCs might as well secrete growth factors that can boost endogenous repair and produce certain chemokines thereby attracting endogenous progenitor cells to the lesion to boost endogenous repair. This hypothesis on secretory effects of stem cells is supported by previous work of our group on transplantation of mesenchymal stem cells (MSCs) which induced repair of HI brain injury by supporting proliferation and differentiation of endogenous stem cells into new neurons and oligodendrocytes without actual integration of the MSCs into the brain (Donega et al., 2014; van Velthoven et al., 2010). In line with this finding, it would be interesting to test whether the secretome of NSC as such could have a regenerative effect on neonatal HI brain injury. For example it would be relevant to investigate the effect of transplantation of NSC-derived exosomes, as these vesicles have been already proposed as a new tool to repair brain damage (Braccioli et al., 2014; Xin et al., 2013). Most recently, Ophelders et al., showed that MSC-derived exosomes were beneficial in an ovine model of HI (Ophelders et al., 2016).

When comparing our previous work on MSC transplantation to the current work on NSC transplantation there are a few pros and cons to either of the transplanted cell types. Both types of stem cells seem to have a potent regenerative capacity on HI brain damage, although

intracranial injection of similar amounts of MSCs locally in the damaged hippocampus seem to have less effect on improvement of motor behavior than NSC transplantation in the current study (van Velthoven et al., 2011; van Velthoven et al., 2012). Transplanted NSCs themselves seem to integrate into the brain and form new neuronal precursors, which could, at least partially, contribute to repair of damaged tissue. Our previous studies using MSC therapy have shown that MSCs are short-lived after transplantation in the brain and do not integrate (Donega et al., 2014; van Velthoven et al., 2011). Our previous work together with the current study show that transplantation of both stem cell types reduces the neuro-inflammatory response probably due to secretory effects of the stem cells and that both types of stem cells can boost endogenous repair (Donega et al., 2014). A possible drawback of the current study with NSCs is that we used an invasive route of administration which makes it hard to translate to the clinical setting. Administration through other routes (e.g. intravenous or intranasal) might be more attractive if proven as efficient. Our current data show that NSCs are capable of migrating from the contralateral hippocampus towards the damaged ipsilateral site. This observation illustrates the great potential of applying NSCs at sites more distant from the lesion, e.g. via a non-invasive route via the nose, as we have shown to be effective for MSCs. Studying the potential of intranasal NSC transplantation will be subject of our future research. Furthermore, it would be of great interest to study the effect of delayed NSC treatment in combination with hypothermia, the latter being the only efficacious treatment in the clinic at present.

In addition, a possible disadvantage of NSCs over MSCs is the fact that NSCs are harder to isolate and expand when compared to MSCs. MSCs can relatively easy be isolated from bone marrow or adipose tissue of the patient (Hass et al., 2011), whilst NSCs have to be isolated from post-mortem tissues or derived from induced pluripotent stem cells (Liu, 2013). We show here as proof of principle the use and efficacy of embryonic allogenic NSCs. For human application the use of embryos as source of transplantable cells is ethically impeded, therefore future efforts should focus on different sources of NSCs (e.g. the above mentioned induced pluripotent cell-derived NSCs). Further studies in that direction are required in order to bring NSC transplantation closer towards a clinical application for neonatal patients suffering from HI brain injury.

Methods

NSCs

NSCs derived from embryonic day 14.5 CD-1 mice were purchased at R&D Systems (Minneapolis, MN) and cultured as neurospheres in DMEM:F12 medium (Thermo Scientific, Waltham, MA) supplemented with 50x B-27 (Thermo scientific) plus 20 ng/mL EGF and 20 ng/mL bFGF (both Peprotech, Rocky Hill, NJ) and antibiotics (Thermo Scientific). Prior to administration, neurospheres were dissociated into single cells and resuspended in PBS. For

in vivo tracing, NSCs were labeled with PKH26 Red fluorescent cell linker kit according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). NSCs were characterized nestin-positive at the time of transplantation indicating progenitor state.

NSC conditioned medium (CM) was generated by growing neurospheres for 72 hours. The medium was then filtered with a 0.2 µm sterile filter to remove the NSCs.

Animals

All experiments were performed in accordance to international guidelines and approved by Experimental Animal Committee Utrecht (DEC, University Utrecht, Utrecht, Netherlands). Briefly, 9 day old (P9) C57BL/6J mice underwent HI by permanent right carotid artery occlusion under isoflurane anesthesia (4% induction, 1.5% maintenance) followed by 45 min exposure to 10% oxygen (Nijboer et al., 2008). This procedure results in unilateral damage to hippocampus, neocortex, and striatum (van der Kooij et al., 2010). Sham-operated control animals underwent anesthesia and incision only. In total 43 pups of both genders from 8 different litters were randomly distributed amongst every experimental group. No significant gender differences were identified at any of the measured parameters. All analyses were performed in a blinded set-up.

At day 10 after HI, $1x10^5$ NSCs resuspended in 2 µl PBS or vehicle (2 µl PBS) were injected under isoflurane anesthesia at 2 mm caudal to bregma, 1,5 mm right from midline, and 4 mm below dural surface in order to reach the ipsilateral hippocampus. For the migration experiment, similar volume and numbers of NSCs were injected into the *contralateral* hippocampus (similar coordinates but left from midline).

Animals were sacrificed at day 13 (3 days post-treatment; NSC n=6), day 15 (5 days post-treatment; SHAM n=3, VEH n=3, NSC n=5) or day 56 (46 days post-treatment; SHAM n=9, VEH n=9, NSC n=9) after HI by overdose pentobarbital. Animals were transcardially perfused with PBS followed by 4% formaldehyde. Brains were collected and post-fixed in 4% formaldehyde.

Cylinder rearing test

The cylinder rearing test (CRT) was used to assess forelimb use asymmetry as described before (Schallert et al., 2000; van der Kooij et al., 2010). Animals were tested in the CRT at 10 days (prior to NSC treatment), 28 days and 56 days after HI. In short, mice were individually placed in a Plexiglas cylinder and observed for 3 minutes. The forepaw that was used to contact the cylinder wall during a weight-bearing full rear was scored as left (L; impaired), right (R; non-impaired), or both. Non-impaired (R) paw preference was calculated as follows: [(R− L)/(L+R+ both)] × 100% (van der Kooij et al., 2010; van Velthoven et al., 2010). Inclusion criterion was a minimum of 10 weight-bearing movements in 3 minutes. The following

animals were excluded as they did not meet this inclusion criteria: 1 SHAM, 2 VEH at 10 days; 2 NSC at 28 days, 3 VEH at 56 days. The test was performed by a trained observer blinded to treatment.

Histology and immunohistochemistry

Brains collected at 56 days post-HI were embedded in paraffin. Coronal sections (8 µm) were cut at hippocampal level (-1.8 mm from bregma) and stained with hematoxylin-eosin (HE) (Klinipath, Duiven, the Netherlands). HE staining was used to assess ipsilateral hemispheric area loss. Other coronal sections were immunohistochemically stained with mouse anti-microtubule-associated protein 2 (MAP2) (Sigma-Aldrich, 1:1000), mouse anti-myelin basic protein (MBP) (Sternberger Monoclonals, Lutherville, MD, 1:1600), or mouse anti-synaptophysin (Abcam, 1:20) followed by biotinylated horse anti-mouse antibody (Vector Laboratories, Burliname, CA) and revealed using Vectastain ABC kit (Vector Laboratories). Stainings for MAP2 and MBP were used to assess neuronal damage and white matter damage respectively. Synaptophysin, which stains for small synaptic vesicles was used to assess neuronal integrity and axonal sprouting/synaptogenesis. HE-positive or MAP2-positive areas in the ipsilateral and contralateral hemisphere were outlined manually with image processing tools in Adobe Photoshop. The area of MBP staining in both hemispheres was quantified using Image J software (Schneider et al., 2012). Ipsilateral area loss (HE), MAP2 loss or MBP loss was calculated as [1-(ipsilateral positive area/contralateral positive area)] * 100%. For synaptophysin, photographs were taken in the CA3 and DG areas of the hippocampus with a Zeiss Axio Lab A1 microscope and Icc5 camera (Carl Zeiss, Oberkochen, Germany).

Primary microglia experiments

Primary mixed glial cell cultures were obtained of P0-2 Sprague Dawley rat brains as described by Chen et al.(Chen et al., 2007). Glial cultures were kept for at least 10 days in vitro before microglia isolation. Mixed glial cell cultures were shaken on an orbital shaker for 1 hour at 200 rpm to detach microglia. Microglia were plated at 300.000 cells per well onto poly-L-ornithine-coated 24-well plates in DMEM with 10% FCS and antibiotics. After 24 hours, 500 µL of NSC-conditioned medium (CM) or control medium (non-conditioned NSC growth medium; CTR) were added to the cultures for 24 hours. Supernatants were collected and analyzed for TNFα production by using ELISA according to manufacturer's instructions (Ucytech, Utrecht, The Netherlands).

Immunofluorescence

Brains collected at 13 and 15 days post-HI were cryoprotected in grading 30-50% sucrose and embedded in OCT freezing medium (Tissue-Tec, Sajura Zoeterwoude, The Netherlands). Coronal cryosections (10 µm) were incubated with rabbit-anti-Iba1 (Wako Chemicals, Osaka, Japan, 1:500), mouse anti-GFAP (Acris antibodies, San Diego, CA, 1:100), goat

anti-DCX (Santa Cruz Biotechnology, Dallas, TX, 1:100) or mouse anti-Nestin (BD Biosciences, Breda, The Netherlands, 1:100) followed by incubation with secondary antibodies donkey anti-rabbit AF680, donkey anti-mouse AF488, donkey anti-goat AF680 (all Thermo Scientific, 1:250). Nuclei were counterstained with DAPI (Sigma Aldrich, 1:2000) and sections were mounted with FluorSave reagent (Merck Millipore, Darmstadt, Germany). Fluorescent confocal images were captured with an AxioCam MRm (Carl Zeiss, Sliedrecht, The Netherlands) on a LSM 700 confocal microscope (Carl Zeiss). Iba1 and GFAP intensity was quantified using Image J software by measuring the staining intensity in 5 different fields of the hippocampus.

For staining of the microglia cultures, cells were fixed in 4% formaldehyde and stained with rabbit-anti-Iba1 (1:500), followed by incubation with donkey-anti-rabbit AlexaFluor-555 (Thermo Scientifics). Nuclei were counterstained with DAPI. Images were acquired using the EVOS FL Cell Imaging System (Thermo Scientifics). The expression level of Iba1 per cell was determined using Image J software.

Statistics

Data are expressed as mean ± SEM and regarded statistically significant if $p < 0.05$. Data were analyzed using one- or two-way ANOVA with LSD post-test. For the conditioned medium experiments, two-tailed, unpaired Student's t-test was used. Outliers were determined with the Grubbs' test.

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CHAPTER 5

Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage.

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Abstract

Mesenchymal stem cells (MSCs) have been shown to improve outcomes after neonatal hypoxic-ischemic (HI) brain injury possibly by secretion of growth factors stimulating repair processes. We investigated whether MSCs, modified to secrete specific growth factors, can further enhance recovery. Using an in vitro assay, we show that MSC-secreting brain derived neurotrophic factor (BDNF), epidermal growth factor-like 7 (EGFL7), persephin (PSP), or sonic hedgehog (SHH) regulate proliferation and differentiation of neural stem cells. Moreover, mice that received an intranasal application of 100,000 MSC-BDNF showed significantly improved outcomes as demonstrated by improved motor function and decreased lesion volume compared with mice treated with empty vector (EV) MSCs. Treatment with MSC-EGFL7 improved motor function but had no effect on lesion size. Treatment with MSC-PSP or MSC-SHH neither improved outcome nor reduced lesion size in comparison with MSC-EV–treated mice. Moreover, mice treated with MSC-SHH showed even decreased functional outcomes when compared with those treated with MSC-EV. Treatment with MSC-BDNF induced cell proliferation in the ischemic hemisphere lasting at least 18 days after MSC administration, whereas treatment with MSC-EV did not. These data suggest that gene-modified cell therapy might be a useful approach to consider for treatment of neonatal HI brain damage. However, care must be taken when selecting the agent to overexpress.

Introduction

Transplantation of mesenchymal stem cells (MSCs) into both neonatal and adult ischemic brain injury models has been reported to promote endogenous repair processes, to reduce lesion size, and to improve functional outcomes.[1,2,3,4,5,6,7] Although it has been shown that MSCs can differentiate into cells of the neuronal or glial lineage, their beneficial effects are not likely to be due to replacement by MSCs of lost cells. Transplanted MSCs rather promote repair of damaged brain tissue via release of trophic factors stimulating endogenous repair processes such as neurogenesis, angiogenesis, and synaptogenesis.[3,8]

In vitro, there is a low-level basal secretion of neurotrophins and growth factors by MSCs. However, in vitro culture of MSCs with ischemic brain extracts induces the expression of several growth factors and cytokines.[1,9,10] In this respect, it is of interest that the type and extent of injury may guide the expression pattern of these MSC-derived growth and differentiation factors after transplantation into the brain.[8,9]

Perinatal hypoxia-ischemia (HI) often leads to permanent brain damage, causing neurological deficits such as cerebral palsy, mental retardation, and seizures.[11] We have previously shown that upon transplantation of MSCs after perinatal HI, graft survival was limited to only ~22% of MSCs surviving until 3 days after transplantation, and 18 days after transplantation, only ~1% of transplanted MSCs were still detectable.[8] However, transplanted MSCs were shown to be capable of extensively modulating growth factor production in the brain.

Following the transplantation of MSCs, there is an increased gene expression of factors involved in cell proliferation/differentiation. These specific MSC-induced changes in growth factor environment may have the potential to regulate repair processes in the ischemic brain.

In this article, we investigated whether the overexpression of brain derived neurotrophic factor (BDNF), epidermal growth factor-like 7 (EGFL7), persephin (PSP), or sonic hedgehog (SHH) in MSCs can further reduce HI brain damage. These factors were chosen based on their capacity to act on different repair processes. BDNF is an all-round neurotrophic factor stimulating diverse processes such as neurogenesis, angiogenesis, and synaptic plasticity.[12,13] Furthermore, it has been shown that infusion of BDNF can significantly improve outcomes after adult cerebral ischemia.[13] EGFL7, also known as vascular endothelial statin (VE statin), Zneu1, or Notch4-like protein, is a secreted antagonist of Notch receptor–mediated signaling that is expressed by endothelial cells, several progenitor cell populations, and a subset of neurons in the adult brain.[14,15] Notch signaling is involved in a wide variety of cellular processes in the developing nervous system, including cell proliferation, differentiation, and apoptosis. By inhibiting Notch signaling, EGFL7 has the potential to increase proliferation of progenitor cells and drive neuronal differentiation. PSP is a member of the TGF-β family

and mainly known for its neuroprotective properties. By engineering MSCs to express PSP, distressed neurons in the ischemic lesion could potentially be protected. SHH is a molecule that, during development, drives migration and differentiation of neural progenitor cells toward neurons and oligodendrocytes.[16,17,18] Neonatal HI causes severe damage, and SHH has a strong potential to stimulate the formation of new oligodrendrocytes, thereby possibly attenuating the deleterious effects of HI brain damage.

Materials and methods

Adenoviral vectors

Adenoviral vectors (pAd-HM41-K7; Alphagen, Yokohama-shi, Japan) carrying the gene for polylysine-mutated fiberknot were constructed as described previously.[35] Mouse BDNF cDNA was cloned using reverse transcriptase–polymerase chain reaction using total RNA isolated from brain as the template. The identity of BDNF cDNA was confirmed by sequencing and comparing it with the GenBank sequence NM_007540. The mouse BDNF primer sequence was forward 5'-TCTAGACACCCCACCATGACCATCCTTTTCCTT-3', Reverse 5'-TCTTCCCCTTTTAATGGTCAGT-3'. The BDNF cDNA was coupled to an IRES-eGFP sequence derived from pPRIG plasmid by means of polymerase chain reaction to allow labeling of infected cells. The BDNF-IRES-eGFP sequence was inserted into the pShuttle2 vector, which contains a cytomegalovirus promoter, between the XbaI and AflII sites resulting in the pShuttle2-BDNF-IE plasmid. pAd-HM41-K7-BDNF-IE was constructed by ligation of I-CeuI/PI-SceI-digested pShuttle2-BDNF-IE with I-CeuI/PI-SceI-digested pAd-HM41-K7.

Adenoviral vectors carrying mouse EGFL7 (pAd-HM41-K7-EGFL7-IE), PSP (Pspn; pAd-HM41-K7-PSP-IE), or SHH (pAd-HM41-K7-SHH-IE) were constructed as described above. The identity of EGFL7, Pspn, and Shh cDNA was confirmed by sequencing and comparing it with the GenBank sequences NM_008486, NM_008954, and NM_009170, respectively. Primer sequences used are as follows:

EGFL7 Forward 5'-CTCTAGACCACCATGCAGACCATGTGGGGC-3', Reverse 5'-CGGCGC GCCCAGATCTTTTTTGCAGGA-3'; Pspn Forward 5'-GGCGCG CCGCCACCAC AGCCAC AAGC-3', Reverse 5'-TCTAGAATGGCTGCAGGAAGACTT-3'; and Shh Forward 5'-CTCTAG ACCACCATGCTGCTGCTGCTGGCCAGATGTT-3', Reverse 5'-CGGCGCGCCGCT GG ACTTGA CCGCCATTCCCAAGC-3'. An EV control was generated and consisted only of the IRES-eGFP sequence insert into the pShuttle2 plasmid.

Virus particles were generated by transfection of PacI-digested pAd-HM41-K7-BDNF-IE into 293 cells with Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Virus particles were purified using Adeno-X virus purification kit (Clontech, Mountain View, CA), and titer was determined by using Adeno-X Rapid Titer Kit (Clontech). Before being used, the titer of the virus particles is determined, and virus stocks are examined for potential contamination with replication-competent viruses.

Mesenchymal stem cells

Mouse C57Bl/6 MSCs (Life Technologies) were purchased and cultured according to the manufacturer's instructions. Cells were positive for CD29, CD34, CD44, and Sca-1 and negative for CD117 (Figure 1e).

For adenovirus-mediated gene transfection, MSCs were seeded at a density of 3×10^6 cells per 25 cm^2 flask. MSCs were exposed to the virus particles in 7.5 ml Dulbecco's modified Eagle medium (DMEM; Life Technologies) at 37 °C for 2 hours after which the cultures were washed three times with DMEM (Life Technologies) and recultured with normal medium. After infection, the cells were cultured for an additional 24 hours after which transplantation was performed.

Growth factor ELISAs

To determine the most effective MOI (pfu/cell) to modify MSCs, cell culture supernatants were collected 48 hours after in vitro transduction of MSCs at various MOIs. The following commercial ELISA kits were used to quantify the amount of growth factor produced: BDNF (Promega, Madison, WI), EGFL7 and PSP (USCN Life Science, Wuhan, China), and SHH (R&D systems, Minneapolis, MN).

Noncontact coculture of NSCs with modified MSCs

To determine the effects of growth factor hypersecreting MSCs on NSCs, cells were placed in a noncontact coculture. Mouse cortical CD1 NSCs were purchased and allowed to expand according to the manufacturer's instructions (R&D systems). NSCs were cultured as neurospheres in DMEM:F12 with B27 supplement (Life Technologies) containing 20 ng/ml of bFGF and EGF (Peprotech, Rocky Hill, NJ) until start of coculture with MSCs. One day before start of coculture, NSCs were plated on poly-l-ornithine and laminin (Sigma-Aldrich, Steinheim, Germany) coated 24-well plates. In another plate, 80,000 modified MSCs were embedded into 0.2% HydroMatrix gel in DMEM with 10% fetal calf serum (Sigma-Aldrich), in order for the MSCs to be cultured in their optimal culture medium, and placed into transwell inserts (Millipore, Darmstadt, Germany). Next day, transwell inserts containing MSCs were placed in well containing NSCs. At 0, 72, and 120 hours after start of coculture, NSCs were washed with phosphate-buffered saline and fixed in 3.7% paraformaldehyde. To detect proliferating cells, NSCs were incubated with rabbit-anti-Ki67 (Abcam, Cambridge, UK) and goat-anti-rabbit Alexafluor594 (Life Technologies).

NSCs were identified by staining cells with mouse-anti-nestin (BD, San Jose, CA), and differentiation of NSCs toward mature neurons, astrocytes, and oligodendrocytes was analyzed by staining cells with rabbit-anti-βIII-tubulin (Abcam), mouse-anti-GFAP (Cymbus Biotechnology, Southhampton, UK), and rabbit-anti-Olig2 (Millipore), respectively. Primary antibody binding was visualized with goat-anti-mouse Alexafluor488 or goat-anti-rabbit Alexafluor488 (Life Technologies). 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used to visualize cell nuclei. For each condition, three randomly selected high magnification fields were selected and photographed. Three independent experiments were analyzed. Ki67- and Olig2-positive cells were calculated as the percentage of total DAPI-positive nuclei per field. Nestin, βIII-tubulin, and GFAP expression were analyzed by

calculating the total staining intensity per field divided by the number of DAPI-positive nuclei.

Animals

Experiments were performed according to international guidelines and approved by the local experimental animal committee. At postnatal day 9, C57Bl/6J mice underwent HI by right common carotid artery occlusion under isoflurane anesthesia (3% induction, 1% maintenance in O2:N2O (1:1)) followed by exposure to 10% oxygen in nitrogen for 45 minutes. This procedure induced a lesion involving hippocampus, neocortex, and striatum.[36] Sham controls underwent anesthesia and incision only. Pups from at least five different litters were used in each experimental group, and pups of each litter were randomly assigned to all experimental groups taking sex into account in a way that both sexes were equally distributed among experimental groups. Data were obtained from at least two independent experiments. Mortality (~10%) only occurred during or immediately after HI, and there were no sex differences in mortality. All analyses were performed in a blinded set-up.

At 10 days after HI, MSCs or vehicle was delivered intranasally in awake animals. Thirty minutes before MSC or vehicle administration, two doses of 3 µl hyaluronidase (total 100 U, Sigma-Aldrich) in phosphate-buffered saline were applied to each nostril and spontaneously inhaled.[37,38] Subsequently, a total of 5×10^5 MSCs in 12 µl phosphate-buffered saline or vehicle were administered as two doses of 3 µl applied to each nostril.

To evaluate cell proliferation/survival, mice received EdU (50 mg/kg intraperitoneally; Life Technologies) on days 10, 11, and 12 after HI and received BrdU (50 mg/kg intraperitoneally; Sigma-Aldrich) on days 21, 22, and 23 after HI. Animals were sacrificed at 28 days after HI and perfused with 4% paraformaldehyde in phosphate-buffered saline.

Functional outcomes

The cylinder rearing test was used to assess forelimb use asymmetry as described.[2] Mice were placed in a clear Perspex cylinder (80 mm in diameter and 300 mm in height) for 3 minutes. As they vertically reached up to explore the cylinder wall, the forepaw to touch the wall would be registered. The forepaw to contact the wall during a full rear was recorded as left (impaired), right (nonimpaired), or both. During the 3-minute period, at least 15 weight-bearing contacts should be registered or the mouse would be retested 30 minutes later. Paw preference was calculated as ((nonimpaired–impaired)/(total forelimb usage)) × 100%. Interobserver reliability was 0.87 at 21 or 28 days after HI and 0.76 at 10 days after HI.

Histology and immunohistochemistry. To asses gray and white matter damage, coronal paraffin sections (6 µm) were incubated with MAP2 (Sigma-Aldrich) or mouse-anti-myelin basic protein (Sternberger Monoclonals, Lutherville, MD), and binding was visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Brain damage was analyzed at a location equivalent to −1.58 mm from bregma in adult mice by outlining both hemispheres on full-section images using ImageJ software (Rasband WS, ImageJ, NIH, Bethesda, Maryland; http://rsb.info.nih.gov/ij/, 1997–2009). Ipsilateral MAP2 and MBP area loss were calculated as described.[2]

For cell proliferation analysis, sections were incubated with biotinylated sheep-anti-BrdU and rabbit-anti-Ki67 (both Abcam). Visualization was done with AlexFluor-594–conjugated streptavidin and donkey-anti-rabbit AlexFluor488. EdU incorporation was detected by incubating sections in 100 mmol/l Tris containing 0.5 mmol/l CuSO4, 50 mmol/l ascorbic acid, and 10 µmol/l AlexaFluor-594-azide. BrdU-positive or EdU-positive cells were counted in hippocampus and six cortical high magnification fields in three 200 µm spaced sequential sections per brain.

Statistical analysis. All data are expressed as means ± SEM. Functional outcomes measured with cylinder rearing test was analyzed using two-way ANOVA with Fisher's least significant difference posttests. $P < 0.05$ was considered statistically significant. Histological measures were analyzed using one-way ANOVA with Bonferroni posttests. $P < 0.05$ was considered statistically significant.

Results

Characteristics of gene-transduced MSCs

MSCs were transduced with different adenoviral constructs at multiplicity of infections (MOIs) of 200, 500, 1,000, and 2,000 plaque forming units (pfu) per cell. The level of BDNF secretion by MSCs transfected with BDNF (MSC-BDNF) at an MOI of 500 pfu/cell was 83-fold higher than by uninfected MSCs (Figure 1a). The level of EGFL7 secretion from MSC-EGFL7 infected at an MOI of 500 pfu/cell was 51-fold higher than by uninfected MSCs (Figure 1b). The level of PSP secretion from MSC-PSP infected at an MOI of 500 pfu/cell was 43-fold greater than by uninfected MSCs (Figure 1c). The level of SHH secretion from MSC-SHH infected at an MOI of 500 pfu/cell was 82-fold greater than by uninfected MSCs (Figure 1d). Flow cytometric analysis of empty vector (EV)-MSC, MSC-BDNF, MSC-EGFL7, MSC-PSP, and MSC-SHH showed that phenotypic expression was essentially identical to primary MSCs. All gene-modified cells and MSCs with EV had the CD29+, CD34+, CD44+, Sca-1+, CD45−, and CD117− cell surface phenotype (Figure 1e).

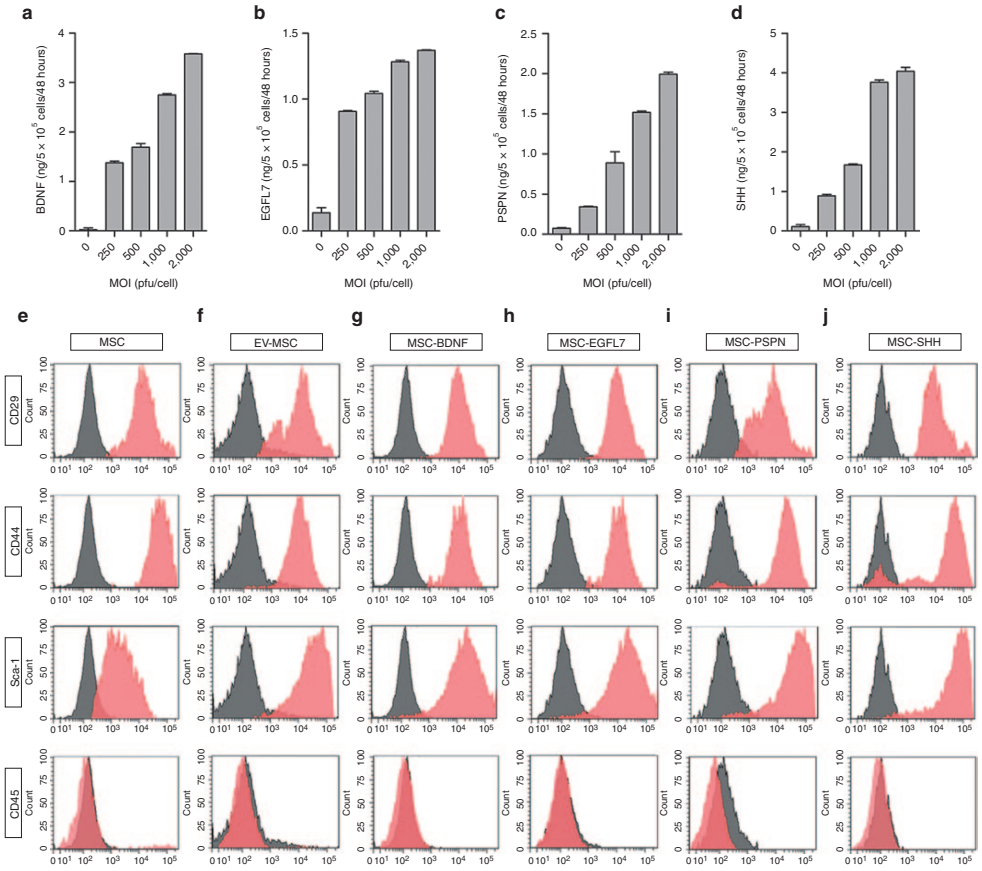

Figure 1: Characteristics of modified mesenchymal stem cells (MSCs).

Secretion of (**a**) brain derived neurotrophic factor (BDNF), (**b**) epidermal growth factor-like 7 (EGFL7), (**c**) PSPN, and (**d**) sonic hedgehog (SHH) by MSCs transfected with pAd-HM41-K7-BDNF, pAd-HM41-K7-EGFL7, pAd-HM41-K7-PSPN, or pAd-HM41-K7-SHH at multiplicity of infections (MOIs) of 250, 500, 1,000, or 2,000 pfu/cell. Data represent mean ± SEM. Flow cytometric analysis of surface antigen expression on (**e**) naïve MSCs, (**f**) EV-MSC, (**g**) BDNF-MSC, (**h**) EGFL7-MSC, (**i**) PSPN-MSC, and (**j**) SHH-MSC. EV, empty vector; PSP, persephin.

In vitro effect of gene-transduced MSCs on NSC proliferation and differentiation

To determine the effect of growth factor secretion by gene-transduced MSCs on the process of neurogenesis following neonatal HI in vitro, we used a noncontact coculture model to examine the effects of gene-transduced MSCs on the proliferation and differentiation of neural stem cells (NSCs). MSCs were seeded into a culture insert and placed in a culture of adherent NSCs. The coculture system was then followed for 5 days during which proliferation and differentiation of NSCs was assessed. At 24 hours after start of culture in the presence of either MSC-EGFL7 or MSC-SHH, more NSCs were Ki67-positive than when cultured in combination with an empty gel or EV-MSC (Table 1 and Figure 2a). MSC-BDNF and MSC-PSP did not stimulate proliferation of NSCs at this time point. Culture

Table 1: Summarized results of effect of modified MSCs on NSC proliferation and differentiation *in vitro*

	Time (hours)	Gel	EV-MSC	MSC-BDNF	MSC-EGFL7	MSC-PSP	MSC-SHH
Ki67	0	16.6 ± 2.2	–	–	–	–	–
	24	13.7 ± 2.5	21.9 ± 0.9	21.0 ± 5.0	36.1 ± 6.7*	23.6 ± 1.4	34.0 ± 6.4*
	72	8.7 ± 2.4	15.9 ± 2.8	35.7 ± 5.5***	20.3 ± 2.5	21.9±2.1	12.5 ± 3.1
	120	8.8 ± 1.3	12.8 ± 2.0	31.5 ± 1.8***	26.6 ± 3.2**	14.9 ± 2.0	16.9 ± 3.5
Nestin	0	168.1 ± 21.3	–	–	–	–	–
	72	88.7 ± 24.0	439.5 ± 29.0	493.7 ± 78.9	385.6 ± 32.8	410.6±75.8	909.9 ± 16.9*
	120	993 ± 134.6	578.3 ± 57.4	1,281 ± 58.1**	832.7 ± 64.3	847.2±112.1	531.9 ± 109.1
β III- tublin	0	0.06 ± 0.02	–	–	–	–	–
	72	2.1 ± 1.1	62.7 ± 1.1	61.8 ± 5.0	54.5 ± 8.3	74.6±3.9	48.2 ± 13.3
	120	7.5 ± 0.8	100.8 ± 4.9	73.1 ± 10.6	110.3 ± 19.8	104.7± 12.7	144.9 ± 19.2*
GFAP	0	0.15 ± 0.1	–	–	–	–	–
	72	0.31 ± 0.3	0.18 ± 0.2	0.02 ± 0.01	0.32 ± 0.2	1.87± 1.2	1.61± 1.3
	120	36.1 ± 28.9	175.3 ± 22.4	251.2 ± 29.1	177.7 ± 33.8	172 ± 29.7	568 ± 49.1***
Olig2	0	12.3 ± 0.4	–	–	–	–	–
	72	10.2 ± 0.3	23.3 ± 3.0	12.82± 1.4	23.1 ± 4.9	19.4±2.2	21.2 ± 5.3
	120	24.2 ± 1.2	29.0 ± 0.1	27.5± 1.6	30.6 ± 0.3	24.7 ± 3.2	29.5 ± 2.0

Data are represented as mean ± SEM.
BDNF, brain derived neurotrophic factor; EGFL7, epidermal growth factor-like 7; EV, empty vector; MSC, mesenchymal stem cell; NSC, neural stem cell; PSP, persephin; SHH, sonic hedgehog.
*$P < 0.05$ vs EV-MSC. **$P < 0.01$ vs EV-MSC. ***$P < 0.001$ vs EV-MSC.

with MSC-BDNF significantly increased the percentage of Ki67-positive NSCs at 72 hours after start of coculture when compared with EV-MSC. Culture of NSCs in combination with MSC-EGFL7, MSC-PSP, or MSC-SHH did not increase proliferation of NSCs. At 120 hours after start of coculture, the percentage of Ki67-positive NSCs was still increased when cultured in combination with MSC-BDNF or MSC-EGFL7. Proliferation of NSCs when cultured with MSC-PSP was not altered when compared with EV-MSC at all time points measured. The effect of modified MSCs on the differentiation potential of NSCs in vitro was determined by analyzing the expression of the markers nestin, βIII-tubulin, GFAP, and Olig2. Culture of EV-MSC with NSCs induced nestin expression when compared with NSCs cultured with empty gel. MSC-SHH significantly increased nestin expression in NSCs at 72 hours in culture compared with EV-MSC (Figure 2b). At 120 hours in culture, MSC-BDNF significantly increased nestin expression when compared with EV-MSC. Culture of NSCs with MSC-EGFL7 or MSC-PSPN did not affect nestin expression in NSCs.

Expression of βIII-tubulin was induced in NSCs after 72 hours in culture with EV-MSC when compared with that of NSCs cultured with an empty gel. At 120 hours in culture, MSC-SHH

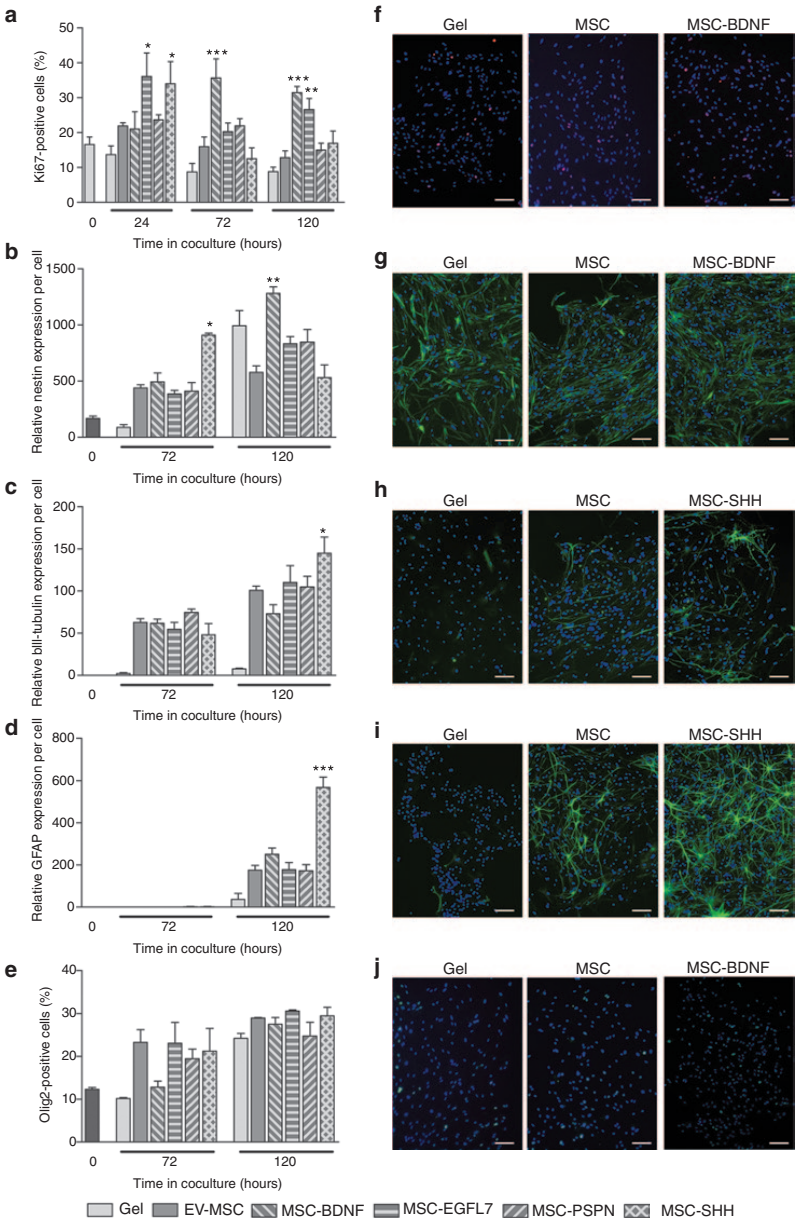

Figure 2: *In vitro* effects of modified mesenchymal stem cells (MSCs) on proliferation and differentiation of neural stem cells (NSCs).

The effects of growth factor hypersecreting MSCs on NSCs were determined using a noncontact coculture system. (a) Ki67 expression was analyzed to determine the effects of MSCs on proliferation of NSC. The effects of MSCs on differentiation of NSCs were determined by examining the expression of the markers (b) nestin, (c) βIII-tubulin, (d) GFAP, and (e) Olig2 in NSC cultures. (f–j) Representative photomicrographs of Ki67, nestin, βIII-tubulin, GFAP, and Olig2, respectively. Data represent mean ± SEM. $n = 3$ for all conditions. *$P < 0.05$ vs EV-MSC. **$P < 0.01$ vs EV-MSC. ***$P < 0.001$ vs EV-MSC. Scale bar represent 100 µm. BDNF, brain derived neurotrophic factor; EGFL7, epidermal growth factor-like 7; EV, empty vector; PSP, persephin; SHH, sonic hedgehog.

significantly increased βIII-tubulin expression in NSCs when compared with EV-MSC (Figure 2c). Culture of NSCs with MSC-BDNF, MSC-EGFL7, or MSC-PSP did not increase βIII-tubulin expression when compared with EV-MSC.

GFAP was hardly expressed in NSCs after 72 hours in culture under all culture conditions. At 120 hours in culture of NSCs with MSC-SHH, GFAP expression was significantly increased when compared with EV-MSC (Figure 2d). MSC-BDNF, MSC-EGFL7, or MSC-PSP did not have any effect on GFAP expression by NSCs. Olig2 expression in NSCs was not affected by coculture with modified MSCs (Figure 2e).

Effect of gene-transduced MSCs on motor function and lesion volume

Mice underwent HI on postnatal day 9 and were treated with gene-transduced MSCs or vehicle on day 10 after HI. At 10, 21, and 28 days after HI, lateralizing motor deficits were quantified using the cylinder rearing test as the preference to use the unimpaired, ipsilateral forepaw when rearing (Table 2 and Figure 3a). Before MSC treatment on day 10, there was no difference between the different treatment groups. Treatment with EV-MSC, MSC-BDNF, MSC-EGFL7, or MSC-PSP significantly reduced forepaw preference on days 21 and 28 after HI when compared with vehicle treatment. Furthermore, MSC-BDNF– or MSC-EGFL7–treated mice showed less lateralizing deficits than mice treated with EV-MSC. Administration of MSC-SHH resulted in decreased impairment at 21 days after HI when compared with vehicle-treated mice. However, at 28 days after HI, forepaw impairment in these mice was

Table 2: Summarized results of in vivo effects of modified MSCs on motor function, lesion volume, and cell proliferation in the hippocampus

		Sham	Vehicle	EV-MSC	MSC-BDNF	MSC-EGFL7	MSC-PSP	MSC-SHH
CRT	10 days	1.93 ± 1.4	10.9 ± 1.1	11.0 ± 1.9	13.1 ± 0.9	12.3 ± 2.0	13.2 ± 2.4	11.1 ± 2.2
	21 days	0.55 ± 1.2	29.2 ± 1.3	20.7 ± 2.0*	11.8 ± 2.0**,φ	13.3 ± 1.0**φ	17.3 ± 0.9"	19.3 ± 2.7*
	28 days	0.43 ± 2.3	29.6 ± 1.9	21.8 ± 1.3	13.0 ± 1.4**,φ	13.0 ± 0.9**φ	18.3 ± 1.4	27.4 ± 1.1
Lesion volume	MAP2	0.42 ± 1.3	39.1 ± 3.5	23.5 ± 3.2*	17.0 ± 2.1**,φ	25.9 ± 5.6	21.9 ± 2.4*	38.1 ± 3.6φ
	MBP 2	54 ± 2.6	42.7 ± 4.4	22.1 ± 2.9*	11.3 ± 3.7**	30.6 ± 4.1	29.1 ± 4.5	27.5 ± 3.3*
Cell proliferation	EdU	9.00 ± 2.1	14.7 ± 1.9	14.4 ± 1.6	25.2 ± 1.3**,φ	27.1 ± 2.9**φ	20.9 ± 0.5	21.4 ± 5.5
	BrdU	10.4 ± 2.3	14.4 ± 2.1	11.3 ± 4.6	26.8 ± 4.9**φ	19.6 ± 4.8	16.7 ± 2.5	21.2 ± 4.0
	Ki67	10.2 ± 1.8	33.4 ± 5.8•	37.0 ± 5.9•	23.5 ± 0.7	29.0 ± 5.0	29.3 ± 2.1	38.4 ± 5.8•

Data are represented as mean ± SEM.
BDNF, brain derived neurotrophic factor; BrdU, bromodeoxyuridine; CRT,cylinder rearing test; EdU, ethynyldeoxyuridine; EGFL7, EV, empty vector; MAP2, myotubule associated protein; MBP, myelin basic protein; MSC, mesenchymal stem cell; NSC, neural stem hedgehog.
*P < 0.05 vs vehicle. **P< 0.01 vs vehicle. φP < 0.05 vs EV-MSC. •P < 0.05 vs Sham.

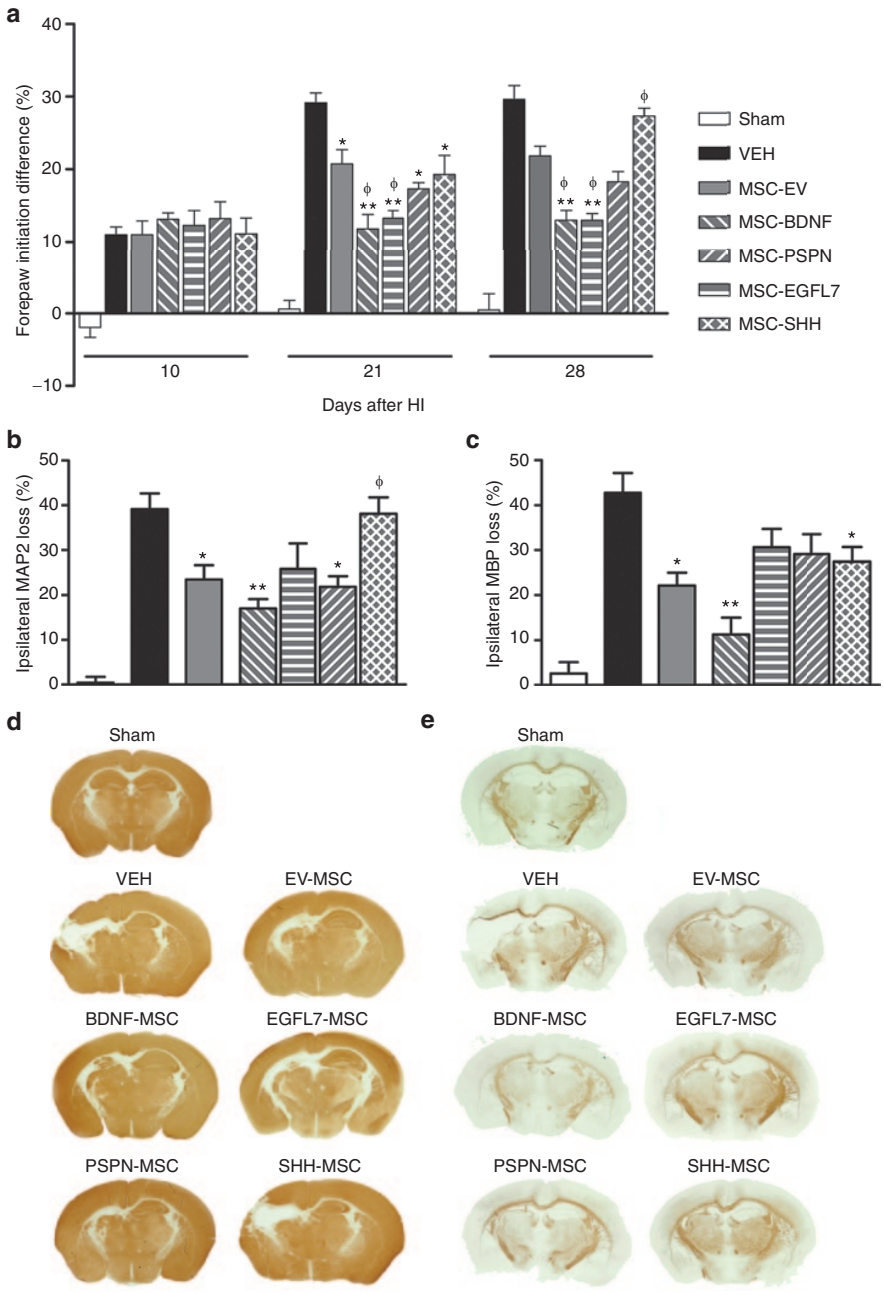

Figure 3: Effect of treatment with genetically modified mesenchymal stem cells (MSCs) on functional outcomes.

Mice received MSCs or vehicle at 10 days after hypoxia-ischemia (HI). (a) Paw preference as a measure of motor disfunction was determined at 10, 21, and 28 days after HI. Quantification of (b) MAP2-positive and (c) MBP-positive area loss expressed as ratio ipsi-/contralateral area. (d,e) Representative photomicrographs of Map2 and MPB respectively. Data represent mean ± SEM. Sham $n = 7$, vehicle $n = 8$, EV-MSC $n = 9$, BDNF-MSC $n = 10$, EGFL7-MSC $n = 9$, PSPN-MSC $n = 10$, and SHH-MSC $n = 10$. $^{*}P < 0.05$ vs vehicle. $^{**}P < 0.01$ vs vehicle. $^{\phi}P < 0.05$ vs EV-MSC. BDNF, brain derived neurotrophic factor; EGFL7, epidermal growth factor-like 7; EV, empty vector; MAP2, microtubule associated protein; MBP, myelin basic protein; PSP, persephin; SHH, sonic hedgehog; VEH, vehicle.

equal to the level of impairment in vehicle-treated mice and significantly worse than mice treated with EV-MSC.

Effect of treatment with modified MSCs on lesion size was determined at 28 days after HI by analyzing the loss of ipsilateral gray (mouse-anti-microtubule-associated protein (MAP2)) and white (mouse-anti-myelin basic protein (MBP)) matter. In vehicle-treated mice, ipsilateral MAP2 area loss was 39.13 ± 3.5% (Table 2 and Figure 3b,d). Treatment with EV-MSC significantly reduced lesion volume when compared with vehicle treatment (23.48 ± 3.2%; $P < 0.05$). BDNF- and PSP-secreting MSCs decreased MAP2 area loss when compared with vehicle treatment (17.08 ± 2.07%; $P < 0.01$ and 21.85 ± 2.38; $P < 0.05$, respectively) but not when compared with EV-MSC. Treatment with MSC-EGFL7 did not significantly decrease MAP2 area loss when compared with vehicle treatment (25.86 ± 5.6%; $P = 0.085$). However, ipsilateral MAP2 area loss in MSC-SHH–treated mice was significantly higher than in MSC-EV–treated mice (38.12 ± 3.64%; $P < 0.05$) and did not differ from vehicle-treated mice.

Treatment with EV-MSC reduced MBP area loss in the ispilateral hemisphere when compared with vehicle treatment (22.13 ± 2.9 vs 42.73 ± 4.4%; $P < 0.05$) (Table 2 and Figure 3c,e). Treatment with MSC-BDNF resulted in a further decrease of MBP area loss when compared with treatment with EV-MSC (11.29 ± 3.7%; $P < 0.01$). Treatment with either MSC-EGFL7 or MSC-PSP did not result in decreased ipsilateral MBP area loss (30.66 ± 4.1 and 29.10 ± 4.5%; $P > 0.05$) compared with vehicle treatment. However, ipsilateral MBP area loss in MSC-SHH–treated mice was significantly smaller when compared with vehicle-treated mice (27.45 ± 3.3%; $P < 0.05$).

Cell proliferation in the ischemic hemisphere after treatment with gene-transduced MSCs

Proliferating cells on days 10, 11, and 12 after HI were labeled using ethynyldeoxyuridine (EdU) (Figure 4a). The number of EdU-positive cells at 28 days after HI as a measure of cell proliferation induced directly after injection of MSCs was increased in the hippocampus of mice treated with MSC-BDNF or MSC-EGFL7 when compared with that of vehicle- or EV-MSC–treated mice (Figure 4b). Treatment with MSC-PSP or MSC-SHH did not result in increased EdU incorporation in the hippocampus when compared with that in vehicle- or EV-MSC–treated mice.

To determine whether there was a lasting effect of growth factor–secreting MSCs on cell proliferation mice were injected with bromodeoxyuridine (BrdU) on days 21, 22, and 23 after HI. Cell proliferation after treatment with BDNF-secreting MSCs was still increased as witnessed by the increased number of BrdU-positive cells in the hippocampus (Figure 4c). Notably, treatment with EGFL7-, PSP-, or SHH-secreting MSCs did not result in a long-lasting induction of

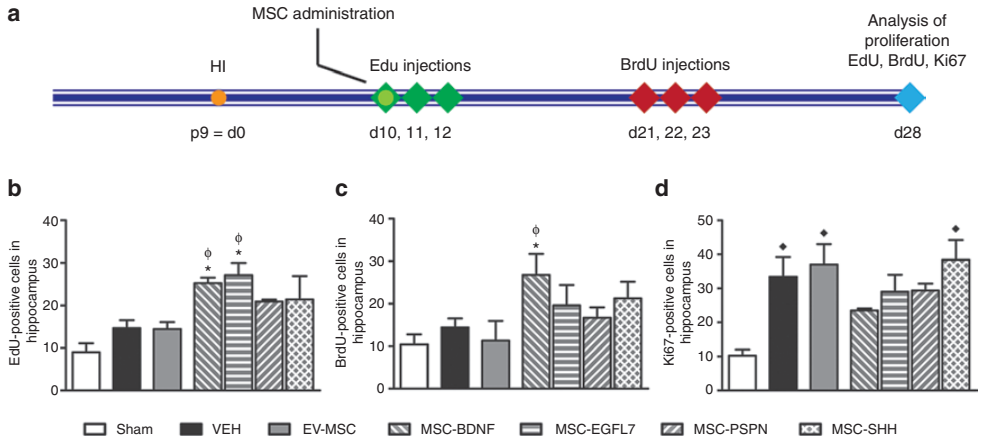

Figure 4: Cell proliferation following treatment with genetically modified mesenchymal stem cells (MSCs).

Cell proliferation marker ethynyldeoxyuridine (EdU) was administered for 3 days starting directly after administration of MSCs, and bromodeoxyuridine (BrdU) was administered on days 21–23. Both were analyzed at 28 days after hypoxia-ischemia (HI). (a) Cell proliferation at 28 days after HI was measured using Ki67. (b–d) EdU-, BrdU-, and Ki67-positive cell numbers were analyzed in hippocampus. Data represent mean ± SEM. Sham $n = 7$, vehicle $n = 8$, EV-MSC $n = 9$, BDNF-MSC $n = 10$, EGFL7-MSC $n = 9$, PSPN-MSC $n = 10$, and SHH-MSC $n = 10$. $^{*}P < 0.05$ vs vehicle, $^{**}P < 0.01$ vs vehicle, $^{\phi}P < 0.05$ vs EV-MSC, $^{\bullet}P < 0.05$ vs Sham. BDNF, brain derived neurotrophic factor; d, day; EGFL7, epidermal growth factor-like 7; EV, empty vector; p9, postnatal day 9; PSP, persephin; SHH, sonic hedgehog.

cell proliferation in the hippocampus. No difference in EdU- or BrdU-positive cell numbers was observed between sham-operated mice or vehicle- and EV-MSC–treated HI mice.
Cell proliferation at 28 days after HI was analyzed in the hippocampus using Ki67. At 28 days after HI, the number of Ki67-positive cells was significantly higher in mice that underwent HI when compared with that in sham controls (Figure 4d). Treatment with EV-MSC or gene-transduced MSCs did not result in increased cell proliferation in the SVZ at 28 days after HI when compared with vehicle treatment.

Discussion

In the present study, we show that intranasal application of modified MSCs after neonatal HI in mice could be beneficial in reducing long-term functional impairment. For this study, we have chosen four different growth factors to be overexpressed in MSCs, which are all known to play a role in proliferation and/or differentiation of NSCs, BDNF, EGFL7, PSP, and SHH.

Using an *in vitro* assay, we first determined the effect of modified MSCs on NSC proliferation and differentiation. The NSCs used in the coculture system are derived from the cortices of 14-day-old mouse embryos (E14). Results from our study indicate that NSCs are most responsive toward MSCs modified to express either BDNF or SHH. Notably, BDNF-secreting MSCs stimulate proliferation of NSCs, and MSC-SHH stimulate differentiation of NSCs toward neurons and astrocytes. In line with our current data, it has been shown earlier

that application of BDNF to E13-derived NSCs promotes their survival and stimulates proliferation.[19,20] Moreover, SHH has been shown to stimulate proliferation of E15 neocortical precursor cells and can promote their differentiation toward all three lineages, i.e., neurons, astrocytes, and oligodendrocytes.[21] These data indicate that the MSCs modified to secrete either BDNF or SHH have the same impact as treatment with pure BDNF or SHH.

MSC-EGFL7 stimulate proliferation of E14 NSCs after 5 days in culture but have no effect on differentiation of NSCs. Recent results studying the effect of EGFL7 on adult NSCs have indicated that EGFL7 reduces the proliferative capacity of the NSCs and induces neuronal and oligodendroglial differentiation.[22] The increase in NSC proliferation induced by EGFL7 in our study could be explained by the age of the NSCs used, because embryonic NSCs react differently to neurotrophic stimuli than adult NSCs. MSCs hypersecreting PSP do not seem to have any effect on proliferation or differentiation of NSCs *in vitro* when compared with EV-MSC.

BDNF is an important neurotrophic factor promoting neurogenesis and angiogenesis. It is neuroprotective, modulates inflammation, and improves synaptic plasticity after ischemic brain injury. Moreover, it has been shown that intracranial infusion of BDNF can reduce the infarct volume after stroke.[13,23,24] Recently, we have shown that BDNF-hypersecreting MSCs have modest beneficial effects on outcomes using a neonatal stroke model.[25] The present study indicates that MSC-BDNF have beneficial effects also in the neonatal HI mouse model. It is the most potent factor in improving motor function, reducing lesion size, and inducing long-term cell proliferation in the ischemic hemisphere. Besides the neuroprotective and neurogenic properties of BDNF, it also plays a role in refinement of developing neuronal circuits.[12,26] The effect of MSC-BDNF might be directed toward refinement of neural circuits, because the neonatal murine brain is still developing during this moment of time. Addition of BDNF to MSCs might lead to more efficient pruning of connections than MSCs alone.

The in vivo data further showed that treatment with MSC-EGFL7 was as effective as treatment with MSC-BDNF in improving motor function. However, this was not combined with reduction in lesion size or increased cell proliferation.

EGFL7 is an antagonist of Notch receptor–mediated signaling. Expression of EGFL7 in NSCs *in vitro* was shown to decrease Notch signaling, thereby reducing proliferation and self-renewal of NSCs.[22] This study also showed that overexpression of EGFL7 in NSCs skewed differentiation of cultured NSCs toward neurons and oligodendrocytes. In our study, we observed that overexpression of EGFL7 by MSCs does not have any effect on differentiation of NSCs but stimulated NSC proliferation after 5 days in culture. In the study of Schmidt *et al*.,[22] NSCs were allowed to differentiate for at least 12 days before analysis, whereas in our study, differentiation analysis was performed after 5 days in culture. The latter could explain

the apparent difference in differentiation of NSCs as 5 days might be too short of an interval to determine the expression of neuronal and oligodendroglial markers. In the neonatal HI model, treatment with MSC-EGFL7 induced cell proliferation in the hippocampus. However, these effects were not lasting, because 1 week after application of the MSCs, cell proliferation was not increased in comparison with EV-MSC treatment. Furthermore, reduction of gray matter damage, as measured by ipsilateral MAP2 staining, was similar after treatment with either EV-MSC or MSC. Likewise, white matter injury, as measured by ipsilateral MBP staining, was not reduced after transplantation of MSC-EGFL7. On the other hand, MSC-EGFL7 caused a greater improvement of motor function than EV-MSC. In a previous study, we reported the beneficial effects of MSCs on the corticospinal tract, which is related to the extent of improvement of motor function.[27] It is possible that besides the previously described effects of EGFL7 on NSCs, it also affects neuronal signaling/communication in white matter tracts, thereby contributing to improved signaling in motor neurons. However, this is purely speculative, and more research is warranted to define the effects of EGFL7 on mature neurons.

PSP is a member of the TGF-β family and closely related to glial-derived neurotrophic factor. Previously, it has been shown that mice lacking PSP are more sensitive to ischemic brain damage following focal ischemia.[28] Damage in these mice could be rescued by a single injection of PSP, and the neuroprotection induced by PSP was 100- to 1,000-fold more effective than glial-derived neurotrophic factor. Furthermore, PSP has been shown to promote survival of midbrain dopamine neurons and motor neurons both *in vitro* and in vivo.[29,30] In our study, adding PSP to MSCs did not result in improved outcomes when compared with EV-MSC. In this study, modified MSCs were transplanted at 10 days after HI injury. Many studies examining neuroprotective agents have indicated that, to be successful, a neuroprotective agent must be administered within a couple of hours after the insult. Because PSP is mainly known for its neuroprotective properties, one could speculate that neuroprotection is not the main mechanism through which MSCs reach their beneficial effects when administered 10 days after onset of injury.

SHH is a powerful factor that stimulates neural progenitor cell proliferation, migration, and differentiation toward neurons and oligodendrocytes.[16,17,18] Theoretically, these properties would make SHH an excellent candidate to be overexpressed in MSCs to treat neonatal HI brain damage. However, our results indicate that addition of SHH to MSCs has detrimental effects on outcomes after treatment for neonatal HI. Using the *in vitro* system, we could detect an early stimulation of proliferation and increased differentiation of NSCs toward neurons and astrocytes when cultured in combination with MSC-SHH. In vivo treatment with MSC-SHH of mice that underwent HI leads to worsened functional outcomes when compared with mice treated with EV-MSC. Furthermore, MSC-SHH–treated mice had significantly more gray matter damage than mice treated with MSC-EV. A recent article by

Sirko *et al*.[31] indicated that SHH elicits stem-cell response in reactive astrocytes following ischemic brain injury. High levels of SHH signaling activate reactive gliosis and trigger a proliferative response in the reactive astrocytes. Although these reactive astrocytes could have beneficial effects in that they protect surrounding tissue from injury, they can also limit repair by creating a glial scar.[32] It could be speculated that by using MSC-SHH to treat neonatal HI, an astrocyte response has taken place that limits MSC-induced repair of brain injury and, therefore, damage evolves as in vehicle-treated mice.

A nonintegrating adenoviral construct was used to overexpress growth factors in MSCs. Adenovrial vectors are known for their immunogenic properties as they continue to express adenoviral proteins in the infected cell. This raises the potential of an immune response against infected MSCs, which could affect their therapeutic potential. However, when we compare the results of the present study with previously published results with naïve MSCs, the reduction in lesion size and improvement of motor function induced by empty adeno vector–transduced MSCs are equal to the effects of naïve MSCs.[1,33] This indicates that an immune response against the adeno-transduced MSCs does not have a major influence on the in vivo effects measured in our present study.

In summary, this study shows that intranasal application of growth factor hypersecreting MSCs have the potential to contribute to the therapeutic potential of MSCs. Repair processes in the brain such as neurogenesis, oligodendrogenesis, and synaptogenesis are regulated via an intricate balance of intra- and extracellular molecules.[34] Meddling in this balance by overexpressing a certain factor could either contribute to repair or impair it. Therefore, care must be taken when selecting the factor to overexpress in the neonatal brain.

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CHAPTER 6

Exosomes: a new weapon to treat the central nervous system.

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Abstract

The potential of exosomes to treat central nervous system (CNS) pathologies has been recently demonstrated. These studies make way for a complete new field that aims to exploit the natural characteristics of these vesicles, considered for a long time as side products of physiological cellular pathways. Recently, however, the biological significance of exosomes has been revaluated and exosomes can now be viewed upon as new relevant functional entities for development of novel therapeutic strategies. In this review we aim to summarize the state of the art on the role of exosomes in the CNS and to speculate about possible future therapeutic applications of exosomes. In particular we will speculate about the use of these vesicles as a substitute of cell-based therapies for the treatment of brain damage and review the potential of exosomes as drug delivery vehicles for the CNS.

Nature of exosomes

Paracrine secretion plays a fundamental role in cell-cell signalling. In eukaryotic cells, carrier vesicles bud from the donor cell membrane and, after being secreted in the extracellular space, subsequently fuse with the intracellular compartment of acceptor cells[1]. A specific subtype of secreted vesicles is represented by exosomes: small spherical vesicles (~50 nm in diameter) limited by a lipid bilayer, enriched in lipids such as cholesterol, ceramide and sphingolipids[2]. The content of exosomes varies ranging from numerous proteins and lipids to mRNAs and microRNAs[3]. Exosomes are derived from late endocytic compartments, known as multivesicular bodies (MVBs). The secretion of exosomes occurs when the MVBs fuse with the plasma membrane[4]. The vesicles diffuse into the intercellular fluids and reach the recipient cells. Here, the exosomal surface molecules will bind to membrane receptors including intercellular adhesion molecule 1 (ICAM1), lymphocyte function-associated antigen 1 (LFA1), and T-cell immunoglobulin domain and mucin domain proteins (TIM1) or TIM4[4]. This interaction might result in the fusion of the exosomes with the recipient plasma membrane or in endocytosis of the exosomes into the recipient cell[4]. In the former case, the exosomal content is directly released into the cytoplasm. Alternatively, endocytosed exosomes fuse with the limiting membrane of the endosome, leading to the release of exosomal content into the cytoplasm of the recipient cell[4]. The recipient cell will then respond to the change in the intracellular compartment depending of the composition of the exosomal content. Exosome secretion is constitutive in many cell types: Epstein–Barr virus-transformed B cells[5], dendritic cells (DCs)[6], macrophages[7], and most tumour cell lines. By contrast reticulocytes[8], T cells[9,10], mast cells[5], and resting B cells[11] increase the secretion of exosomes following activation of a relevant membrane receptor even though the basal level of exosomes is still detectable in resting conditions. Furthermore, a variety of conditions- such as cell activation, radiation, maturation stage of the cell and senescence - can modulate the level of exosomal secretion in different cell types[4,12-15]. The biological role of exosomes has been intensively studied during the last decades, especially regarding their function in the immune system[4,16] and in cancer[17-20]. Remarkably, a growing body of evidence indicates how these vesicles are central players in cell-cell communication in other contexts, like in CNS.

Role of exosomes in the CNS

The study on the role of exosomes in the CNS is a relatively new field: in 2006, the group of Sadoul described for the first time the release of exosomes by cortical neurons *in vitro*[21]. Furthermore, Taylor *et al.* reported the secretion of exosomes by astrocytes[22].

Exosomes can also be found in the cerebral spinal fluid (CSF) in both adult[23] and embryonic animals[24]:The presence of exosomes in the embryonic CSF raises the possibility that they might also play a role in normal brain development.

Interestingly, it has been discovered that exosome-mediated interactions between neurons and glia induce neurite outgrowth and neuronal survival[25]. Concordantly, Xin *et al.* showed how exosomes secreted by mesenchymal stem cells (MSCs) are capable of transferring microRNA-133b into neurons, resulting in the induction of neurite outgrowth[26]. Morel *et al.* showed that neurons secrete microRNA-124a through exosomes: the vesicles are subsequently transported into astrocytes, thereby indirectly increasing GLT1 protein expression[27]. Taken together, these observations support the hypothesis that exosomes mediate cell-cell communication within the CNS.

Given this scenario it is not surprising that exosomes play an important role in the pathophysiology of neurodegenerative diseases. The use of *in vitro* models allowed Rajendran *et al.* to describe the mechanism through which Aβ peptides (responsible for the formation of β-amyloid plaques in the brain of Alzheimer's disease (AD) patients) are secreted within exosomes[28]. Additionally, *in vitro* studies showed that several key members of the secretase family of proteases – involved in the progression of AD - are also found in exosomes[29,30]. Furthermore, the cytoskeletal protein tau, another important player in AD, has been found in exosomes as well[31].

In 2012, Perez-Gonzalez and colleagues published the first *in vivo* evidence of exosome-mediated secretion of amyloid precursor protein carboxyl-terminal fragments from brain cells into the brain extracellular space, evidence that supports the concept of an important physiological role of exosomes in the brain and in AD[32].

The group of Vekrellis *et al.* discovered that also α-synuclein (a central player in the pathogenesis of Parkinson's disease) is secreted in a calcium-dependent manner by exosomes, suggesting a role of the vesicle also in this neurodegenerative disease[33].

Recently, a study reported that exosomes are implicated in brain malignancies: glioblastoma cells are capable of secreting exosomes loaded with mRNAs and microRNAs that are utilized by the recipient tumor cells to contribute to tumor-proliferation and angiogenesis[34]. Moreover, hypoxic conditions are capable of triggering a pro-angiogenic pathway that involves exosome secretion in glioblastoma[35].

We therefore propose that the role of exosomes is of great importance in the biology of CNS diseases, but the question remains whether we can exploit our knowledge about these vesicles to treat CNS diseases, like AD, brain tumors or brain damage.

Exosomes for drug delivery

Targeting drugs to specific cell types has been a major challenge for scientists during the last decades. The necessity for specific delivery vesicles arises from the need to obtain

a selective effect of the drug on the tissue/cell-type of interest without altering the normal physiology of other cell types or tissues. Thus, for a proper application *in vivo* the aim of pioneer research groups has been to develop transport systems that allow drugs to selectively pass biological barriers. With the boom of the small-interfering RNA (siRNA) technology that theoretically allows the modulation of the expression of almost any disease-related gene, the demand of a tailored delivery became even greater. But, despite the intensive research, the practical answers are still insufficient. The number of theoretical possibilities dramatically decreases when we consider the available drug delivery systems directed to the brain.

In fact, since the blood-brain barrier (BBB) isolates the CNS to a certain extent, creating an exclusive biochemical and immunological niche, the delivery of drugs to the brain is a troublesome challenge.

To date there are a few validated non-invasive methods that allow therapeutic molecules (siRNA or more conventional drugs) to reach the CNS. The use of endogenous transport systems allows the therapeutic drug to cross the BBB through (direct or indirect) binding to specific carrier receptors. This can be achieved by conjugating receptor-directed antibodies (e.g. a monoclonal antibody directed against the transferrin receptor[36] entire carrier proteins or chemical groups directly to the drug or to drug-loaded liposomes, small lipidic particles synthetically derived[37]. Although strong optimism has been demonstrated for the pre-clinical success of this approach, it has been shown that the use of liposomes leads to adverse immunogenic reactions[38].

In this field craving for new solutions, the paper published in 2011 by Alvarez-Erviti and co-workers provide the proof-of-concept for the employment of exosomes to deliver siRNA to the mouse brain[39]. The authors exploited autologous murine dendritic cells to produce exosomes. In order to achieve specific targeting, they engineered the dendritic cells to express the exosomal membrane protein Lamp2b fused to the neuron-specific rabies viral glycoprotein (RVG) peptide. With the use of electroporation, they loaded exosomes with GAPDH-directed siRNA and injected the modified vesicles intravenously. The authors were able to observe a specific gene knockdown of GAPDH in neurons, microglia and oligodendrocytes in the brain. To prove the therapeutic potential of the exosome-based delivery system, they loaded the vesicles with siRNA against Beta-site APP Cleaving Enzyme 1 (BACE1), a protein with an important role in the pathology of AD, and achieved a specific and efficient down-regulation of the BACE1 protein. Last but not least, the authors provide an initial indication that engineered exosomes might exhibit a low immunogenicity, since the serum level of pro-inflammatory cytokines *in vivo* did not increase by the therapy. In conclusion, the work of Alvarez-Erviti *et al.* demonstrates not only that exosomes could be used as an efficient delivery tool that is capable of crossing the BBB via a non-invasive route;

they also showed that these particles do not induce an immune response, an obstacle that dampened the development of other delivery vectors, such as liposomes[40].

In view of the fact that that siRNA can be transferred to exosomes to achieve a cell-specific gene knockdown, the next question is: are these vesicles capable of ferrying a different cargo, such as anti-inflammatory or anti-cancer drugs?

Sun *et al.* demonstrated that cancer cell-line derived-exosomes can encapsulate curcumin, a drug exhibiting anti-inflammatory activity[41]. Curcumin, due to its insolubility in aqueous solution and relatively low stability, has a low systemic bioavailability and this constitutes a barrier for its clinical application. Interestingly, Sun *et al.* showed that the incorporation of curcumin into exosomes increases the solubility, the stability and the bioavailability of the drug. Subsequently, they showed through in *vitro* experiments how the curcumin loaded exosomes (Exo-cur) are taken up by RAW 264.7 cells, an immortalized macrophage cell line. Furthermore, to prove that curcumin loaded exosomes (Exo-cur) can effectively exert the anti-inflammatory activity by accumulating in cellular targets, they provided in *vitro* evidence that Exo-cur is capable of inhibiting the inflammatory activity of RAW 264.7 cells upon stimulation with LPS. Consistently, they showed that in an LPS-induced septic shock murine model Exo-cur is taken up by pro-inflammatory circulating macrophages to shift the immune response to an anti-inflammatory M2 phenotype[41].

Exosomes incorporate, stabilize and deliver curcumin to target cells (macrophages), but is this concept applicable to the delivery of drugs to the CNS? The same group tried to solve the issue: Zhuang and colleagues showed how the Exo-cur can be rapidly transported to the mouse brain after intranasal administration, with a very low accumulation of the vesicles in stomach and intestine: the exosomal vesicles transport curcumin to the brain by entering the CNS along the olfactory route, thus allowing the drug to reach the brain in minutes. Zhuang et al. assessed that exosomes are taken up by microglial (60%) as well as non-microglial cells (i.e. neuronal cells and astrocytes) (40%) throughout the whole brain (consistently with the data previously published by Sun *et al.*[41]. Therefore, we can conclude that exosomes cross the BBB, diffuse throughout the brain and deliver the drugs locally. To verify the real therapeutic efficacy of this approach, Zhuang et al. continued their work showing the effectiveness of drug-loaded exosomes. They first showed how intranasally injected Exo-cur is capable of inhibiting brain inflammation and autoimmune responses in a model of experimental autoimmune encephalomyelitis. Moreover, they demonstrated how the intranasal delivered exosomes -loaded with the Stat3 inhibitor JSI-124- inhibit the growth of GL26 tumor cells in a xenograft model of glioblastoma[42].

If development of exosomes as carrier system will be pursued, it could generate a valuable strategy for treatment of CNS diseases that were previously considered as untreatable.

Exosomes and brain regeneration: an alternative to cell-based therapies?

MSCs based therapies are currently being developed to efficiently regenerate tissues damaged by various pathological conditions, such as renal fibrosis[43], lung injuries[44], nucleus pulposus degeneration[45], infarct-induced heart injury[46] and, interestingly, a number of brain pathologies. Promising pre-clinical studies, performed in murine models, show the efficacy of this approach both in the treatment of adult[47] and neonatal brain injuries[48,49]. The capability of these cells to induce regeneration of the injured tissue makes them an attractive candidate for clinical trials: MSCs are not only an effective way to induce brain repair, but they can be collected in an autologous fashion from the bone marrow of the patient, reducing the risk of inducing an allogeneic immune response. Moreover, MSCs immunogenicity is intrinsically low since they do not express HLA-DR antigens. However, as every cell-based therapy, MSC treatments are still to be proven safe (i.e. risk of carcinogenesis).

The concept has recently emerged that MSCs exert their regenerative effect via exosomes. In favour of this theory, recent *in vitro* and *in vivo* studies have reported beneficial effects of MSC-derived exosomes (MSC-Exo). MSC-Exo have been shown to have a beneficial effect on damaged proximal tubular epithelial cell, via transfer of the mRNA encoding the insulin-like growth factor-1 receptor[50].

Lai *et al*. performed a study in a mouse model of myocardial ischemia/reperfusion injury, demonstrating how intravenous injections of MSC-Exo, isolated from human embryo-derived mesenchymal stem cells immortalized with c-Myc, significantly reduced the infarct size after ischemia/reperfusion[51]. In another study, Lee and colleagues demonstrated that MSC-Exo are capable of suppressing the hypoxic pulmonary influx of macrophages and the induction of a pro-inflammatory response in a murine model of hypoxic pulmonary hypertension[52].

Since the efficacy of MSCs in treating the most disparate and diverse diseases points at the existence of an underlying common mechanism such as tissue regeneration, these findings together suggest that MSC-Exo could as well represent the regenerative potential of MSCs to boost endogenous repair mechanisms in models of brain damage[48,49].

Supporting this hypothesis, Xin *et al.* demonstrated that MSC-Exo secreted by MSCs that have been exposed to brain extracts of rats subjected to middle cerebral artery occlusion (MCAo) are capable to induce neurite outgrowth in neural cells. The authors continued by showing that the effect is mediated by the transfer, via exosomes, of microRNA-133b to neurons. Thus, this work suggests that transfer of exosomes from MSCs to neural cells could be one of the pathways how MSCs regenerate the brain after damage. It is important to notice that the transfer of microRNA-133b from MSCs to neural cells is induced only after stimulation of MSCs with MCAo brain extracts[26].

Hence, the cargo of MSC-Exo may change after exposure of MSCs to specific factors. Upon stimulation, the exosome content may vary to include factors that induce a response in the acceptor cells (e.g. neurite outgrowth in the case of neural cells). This suggests that the regulatory state of MSCs at the moment of exosome collection may decide the differential biological activity of the vesicles.

Although Xin and colleagues focused on the MSC-Exo-mediated transfer of microRNA-133b, it is unlikely that this microRNA is the only molecule responsible for the effect these authors observed. Most probably, not one, but a battery of molecules is capable of triggering neurite outgrowth in the neural cells after the uptake of the vesicles. Here lies the potential of exosomes: with their naturally varying cargo, they can have a more powerful and adaptive effect than an artificial vesicle (i.e. liposomes) loaded with a given limited amount of factors. It is not clear yet if other brain cell types than neurons are effectively targeted by MSC-Exo, since the study took only neurons and astrocytes into account.

In the context of brain damage, it is unlikely that MSCs stimulate adult neurons in the CNS to induce regeneration, since these mature and differentiated cells cannot longer proliferate and thus will not be capable of reconstituting the damaged area. Moreover, following a brain insult, affected neurons die in a short time: these cells do not survive long enough to be the potential recipients of MSC-Exo, since MSCs are administered days after the neuronal death occurs[48,49]. For these reasons, it is more logical that MSCs, perhaps through exosomes, activate the endogenous neural stem cells (NSCs). NSCs are resident cells in the subventricular zone of the mammalian brain throughout life[53]. Not only have these cells the potential to replace the damaged cells: it has been shown that they also participate in the endogenous regenerative response following brain damage [54,55]. Hypothetically, MSCs-Exo could be delivered intranasally to mediate the repair of the damaged region[42]. If this hypothesis will be proven true, it would imply a revolution in the field of regenerative medicine. In fact, exosomes can be easily derived using established culturing protocols and isolated through high-throughput techniques in an industrial manner[56]. Due to lack of immunogenicity[42], exosomes could be used in an allogeneic way, consistently speeding up the treatment process, with a possible great improvement in clinical outcome. We propose that delivering exosomes through the intranasal route might be the most optimal route to administer the vesicles to treat cerebral pathologies as we have also shown for MSC in models of brain damage[48,49].

Exosomes could be derived from immortalized human embryonic stem cells-derived mesenchymal stem cells (hESC-MSC), a possibility that has been already explored[51]. These cells can undergo more divisions than normal MSCs, allowing the production of exosomes on a larger scale[56]. However, in order to avoid ethical problems, the use of non-embryonic stem cells to obtain immortalized MSCs may be preferable, such as induced pluripotent stem cells-derived MSCs.

Moreover, MSCs could be stimulated with specific growth and differentiation factors to change the content of MSC-Exo in order to improve beneficial effect, although these factors have still to be identified.

Additionally, MSCs-Exo could be modified in order to enhance their native efficacy and to achieve a specific targeted delivery. For example, to boost white matter regeneration, MSCs could be engineered to express microRNAs that will trigger NSCs differentiation towards the oligodendrocytic lineage[57]. These microRNAs might be taken up by exosomes, enhancing their regenerative properties[26]. To achieve tailored delivery, one way could be to engineer the expression of specific proteins (e.g. antibodies, ligands) on the surface of the exosomes, recognized by receptors selectively expressed on the surface of the target cells. For example, a fusion protein obtained by cloning the gene of an exosomal surface protein in frame with a protein that binds a neuron-specific receptor could increase the affinity for the exosomes towards neurons.

Conclusion

The potential of exosomes in treating CNS diseases has still to be proven, but an increasing number of high quality studies on the subject suggests how in the near future these vesicles may represent a powerful tool. In fact, exosomes may be capable of surpassing the limits of the current conventional drugs to treat the brain pathology. These vesicles could become a valuable tool for those conditions with limited treatment options, like neonatal encephalopathy or stroke, conditions that have already been proven to benefit from MSC treatment. Exosomes derived from allogeneic sources could be administered 'from the shelf' via the intranasal route right away after the insult has been diagnosed. Additionally, the possibility to engineer exosomes suggests how their therapeutic strength could grow exponentially within a few years. However, the clinical safety of exosomes should be fully investigated before being translated to the clinic; the content of exosomes remains to be characterized to allow the inclusion/depletion of factors from exosomes in order to achieve the optimal therapeutic effect. Using proteomics combined with microRNA profiling of these exosomes can lead us to highlight which factors play a role in the therapeutic effect and which molecules could be potentially harmful (i.e. increasing the risk of malignancies).

The development of novel techniques to study these vesicles in relationship to the CNS could accelerate the evolution of exosomes as a clinical weapon. For example, an efficient system to track exosomes *in vivo* could help us to learn more about the final destination of these vesicles, thus providing insight on their cellular targets. Anyhow a lot can be done with the available tools, in particular to answer a number of important questions: is the source of exosomes an important factor to consider (the use of cancer cell-line derived exosomes could be hazardous, since the vesicles may carry endogenous tumor molecules that may

induce malignancies)? Which method should be used to obtain drug incorporation? Which diseases can be effectively treated with naïve or engineered exosomes?

Certainly, the escalating interest in these particles will lead the scientific community to boost the generation of knowledge about the basic mechanism and the therapeutic applications of these tiny powerful vesicles.

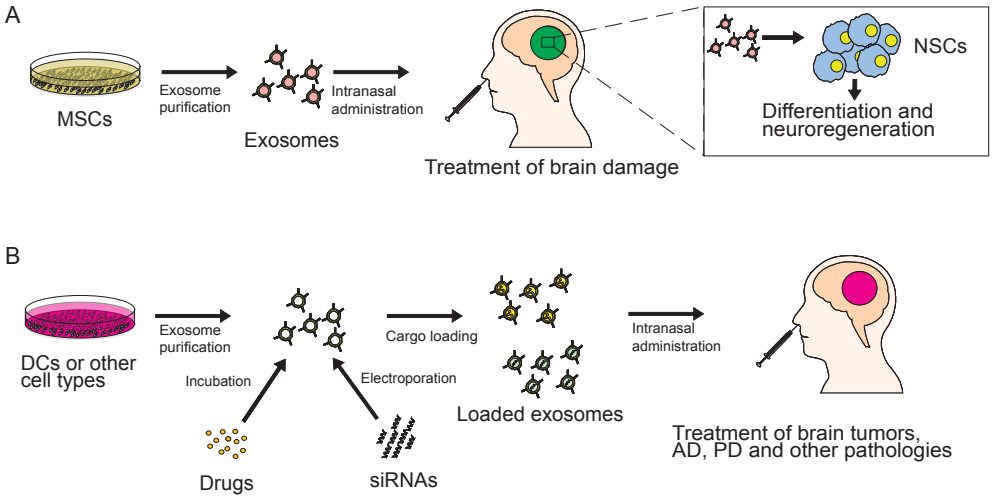

Figure 1: Exosomes as therapeutics.

(**A**) Exosomes are purified from the supernatant of cultured MSCs and administered via the intranasal route. Naive exosomes fuse with neural stem cell (NSC) thereby inducing differentiation of NSC possibly leading to neuroregeneration. (**B**) Exosomes purified from the supernatant of dendritic cells (DCs) or other cell types can be loaded with drugs or siRNAs via incubation or electroporation respectively. The modified exosomes can be administered intranasally to treat pathologies such as brain tumors, Alzheimer's disease (AD) or Parkinson's disease (PD)

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CHAPTER 7

General discussion.

General Discussion

Neurogenesis and gliogenesis are processes that occur during development of the CNS as well as after insult of the nervous system. These tightly regulated processes take place in specialized niches during embryogenesis and adulthood to generate functional cells, starting from defined progenitor cells such as neural stem cells (NSCs). Here we took a multifaceted experimental approach to better understand NSC biology both *in vitro* and *in vivo*. Transcriptional regulation is essential since the expression of specific genes is the key to control timing and fate of the differentiation process. In this thesis two NSC transcriptional regulators, Foxp1 and Sox4 are shown to be crucial for control of neurogenesis and gliogenesis respectively. Mutations in the Foxp1 gene have been associated with speech defects, autism and other intellectual disabilities (Hamdan *et al*., 2010; Le Fevre *et al*., 2013; Lozano *et al*., 2015; Palumbo *et al*., 2013; Sollis *et al*., 2016), as well as being defined as necessary for neurogenesis (Bacon *et al*., 2015). In the work presented in this thesis, we sought to define the molecular mechanisms mediated by Foxp1 that regulate NSC differentiation. Sox4 has been described as inhibitor of gliogenesis and myelination in oligodendrocyte precursor cells (Bartesaghi *et al*., 2015; Hoser *et al*., 2007; Potzner et al., 2007). In this context we investigated the role and molecular mechanisms underlying Sox4-mediated regulation of oligodendrogenesis. The use of cellular strategies to repair CNS insults has been widely investigated in recent years (Daadi *et al*., 2010; Donega *et al*., 2013; Park *et al*., 2002; Titomanlio *et al*., 2011; van Velthoven *et al*., 2010c). Here we further evaluate the therapeutic potential of NSCs and MSCs to treat perinatal hypoxic-ischemic brain damage (HI). Moreover, the hypothesis of the use of exosomes as a cell-free alternative as a therapeutic option upon brain insults and HI is here examined.

Foxp1 promotes NSCs differentiation by regulating the Notch pathway

In the course of corticogenesis the radial glial cells, considered embryonic NSCs, migrate from the ventricular zone (VZ) towards the cortical plate (CP). During this process they differentiate giving origin to the neurons, astrocytes and oligodendrocytes that comprise the cortex (Fishell and Kriegstein, 2003; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). Amongst the different pathways regulating NSC differentiation, the Notch pathway is widely recognized as a pivotal factor that induces neural progenitor maintenance and regulates NSC fate decision both in the embryonic and adult compartment (Ables *et al*., 2011; Louvi and Artavanis-Tsakonas, 2006). During embryonic neural development, Foxp1 has been proposed to promote neuronal migration and morphological development, as well as differentiation of medium spiny neurons (Li *et al*., 2015; Precious *et al*., 2016). Moreover, Foxp1 has been associated with an autism-associated subset of genes that are expressed in the hippocampus and striatum, and heterozygous Foxp1+/- mice have been reported to exhibit vocal communication defects (Araujo *et al*., 2015). Additionally, conditional deletion of Foxp1 in Nestin-positive cells has been shown to induce autism-like behavior in mice, accompanied by structural defects in the region of the postnatal striatum (Bacon et al., 2015). However, the role of Foxp1 has not been

well described during corticogenesis. In **Chapter 2** we demonstrated that Foxp1 regulates embryonic NSC differentiation at least in part by repressing Jag1 expression. It would now be relevant to address whether changes in Foxp1 expression levels in embryonic NSCs are causally linked to development of such diseases, for example by conditionally deleting Foxp1 in these cells during murine embryogenesis and then following neurological development postnatally. Supporting this hypothesis, it has been demonstrated that one particular autism-linked FOXP1 mutation (V423H) disrupted both the nuclear localization and the transcriptional repression capacity of FOXP1 in HEK293 cells. Hence, reduced functionality of FOXP1 could be one of the underlying causes in the establishment of autism (Lozano *et al*., 2015). Additionally, Chien *et al*. showed that a cohort of patients suffering from autism spectrum disorders presented elevated FOXP1 expression (Chien *et al*., 2013). This raises the possibility that augmented FOXP1 levels might also induce aberrant neural development. In our *in vitro* study we observed how overexpression of Foxp1 in NSCs induced increased number of neurons and astrocytes. While this effect could appear advantageous, an increased number of neurons during embryonic development could potentially lead to dysfunctional neuronal circuits, leading to neurodevelopmental conditions such as autism. A previous study from our group showed that Foxp1 expression is regulated by the transcription factor FoxO3. Foxp1 was also shown to inhibit a subset of FoxO3 induced genes through a negative-feedback mechanism (van Boxtel *et al*., 2013). Interestingly, FoxO3 has been demonstrated to be required for NSC maintenance through the induction of a set of genes inhibiting differentiation (Renault *et al*., 2009). It is therefore possible that FoxO3 might also increase Foxp1 expression in NSCs which subsequently mediates suppression of FoxO3-induced genes that hinder differentiation Similarly, Kim *et al* have shown that FoxO1, functionally homologous to FoxO3, is also essential for maintenance of maintaining the long-term proliferative capacity of NSCs (Kim *et al*., 2015). Interestingly, the authors also suggest that FoxO1 and Notch signalling are functionally connected, since FoxO1 induces the expression of Hes1, a transcription factor that mediates the effects of Notch signaling (Kim *et al*., 2015). If Foxo1 induced Foxp1 in NSCs, this could be a way to ultimately repress Notch signalling by targeting Jag1. Given the fundamental function of Notch signalling in maintaining neural progenitors, the finding that Jag1 expression is directly regulated by Foxp1 binding to its promoter represent a novel mechanism through which NSC differentiation can be regulated. However, our data show that the regulation of Jag1 by Foxp1 is important for the neural fate specification of NSCs, while it is dispensable for astrocyte formation. Frizzled 1 (Fzd1), a receptor of the Wnt signaling, has been demonstrated to promote generation of neurons and to inhibit astrocyte formation from adult hippocampal progenitors (Mardones *et al*., 2016). We observed that Fzd1 was upregulated upon Foxp1 KD and bound by Foxp1 respectively in both our RNA-seq dataset and ChIP-seq. It is therefore possible that Fzd1 repression by Foxp1 is essential for astrocyte differentiation. The Wnt pathway has been shown to be important in the homeostasis of NSCs (Lee *et al*., 2000; Zechner *et al*., 2003). We found that Foxp1 represses Fzd1 expression by directly binding to its promoter **(Figure 1A and 1B)**. Inhibition of Wnt pathway by the addition of the Wnt inhibitor

IWP2 was sufficient to rescue reduced astrocyte differentiation after Foxp1 knockdown (KD) NSCs **(Figure 1C and 1D)**. Although these preliminary data must be further validated, this could explain how Foxp1 can control both astrocyte differentiation: by repressing Fzd1 expression, and neuronal specification: by inhibiting Jag1 expression. Dll3, another Notch family ligand, has been shown to inhibit neurogenesis in the developing brain (Zhao *et al*., 2009). We also observed an increase in Dll3 expression upon Foxp1 KD, as well as Foxp1-binding to the Dll3 promoter (data not shown). However, Dll3 has not been well characterized within the Notch pathway, and reports show Dll3 as both inducing and repressing Notch activation (Ladi *et al*., 2005; Zhao *et al*., 2009). Due to the uncertain role of Dll3 we chose not to pursue its functional characterization in relation to Foxp1 in NSCs. Tubby-like protein 3 (Tulp3) is expressed by neurons, and Tulp3 mutant mice exhibited neural tube closure, coincident with a decrease

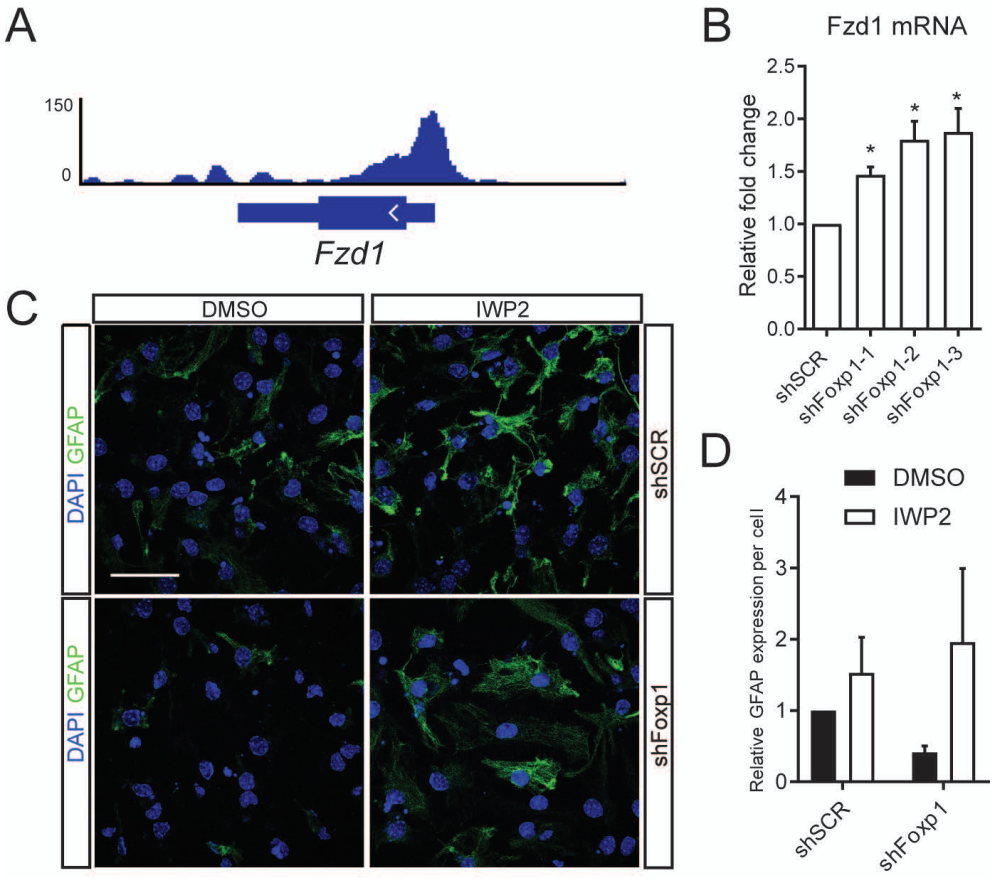

Figure 1: Foxp1 represses Fzd1 expression in NSCs.

(**A**) Foxp1 chromatin immunoprecipitation (ChIP)-sequencing profile around the genomic locus of *Fzd1*. (**B**) Measure of Fzd1 mRNA levels upon Foxp1 KD (n=2). *=p<0.05. (**C**) Foxp1 KD NSCs were differentiated for 5 days in presence of the Wnt inhibitor IWP2 or DMSO. Representative confocal pictures showing the expression of the astrocyte marker GFAP (green). DAPI co-stained in blue (bar=50 µm). (**D**) Quantification of C (n=2). Error bars represent SEM.

of βIII-tubulin positive neurons in the hindbrain (Ikeda *et al*., 2001). This suggests that Tulp3 promotes neurogenesis. We found that *Tulp3* was upregulated in absence of Foxp1, and the Tulp3 promoter was observed to be bound by Foxp1 (data not shown). Although Tulp3 upregulation does not explain the deficit in neurogenesis caused by the depletion of Foxp1 in NSCs, it would be interesting to study whether the regulation of Tulp3 by Foxp1 has any major functional consequences in NSC differentiation.

Our data also show the expression of two different isoforms of Foxp1 in NSCs: Foxp1A and Foxp1C. These isoforms share the same mRNA and the smaller isoform (Foxp1C) is translated from a differential start codon, thus lacking the Q-rich domain (Shu *et al*., 2001). In our work we were unable to address the question as to whether these two isoform have specific roles, since the Foxp1 shRNAs utilized deplete both isoforms. To evaluate a potential isoform-specific role for Foxp1, it would be relevant to generate genetically modified NSCs which lack the differential start codon of Foxp1C, for example through the use of tools such CRISPR/Cas9. Foxp1 has been shown to both homo- and heterodimerize with Foxp2 and Foxp4, which are found co-expressed in distinct areas of the brain (Chen *et al*., 2013; Mendoza *et al*., 2015; Teramitsu *et al*., 2004). It is likely that Foxp1C, which misses the Q-rich domain, is not able to dimerize, therefore exerting a different role than Foxp1A. In conclusion **Chapter 2** demonstrates how Foxp1 induces embryonic NSC differentiation by repressing the expression of Jag1 and possibly Fzd1 (see **Figure 3**). This study sheds light on a novel mechanism of relation to NSC differentiation during development as well as after cerebral injury, which might lead to the development of new tools to treat neurological conditions.

Sox4 inhibits oligodendrocyte differentiation of NSCs

NSCs can give rise to neurons, astrocytes and oligodendrocytes; the lineage choice is fine-tuned by several pathway, amongst which the Notch pathway has been well characterized (Ables *et al*., 2011; Louvi and Artavanis-Tsakonas, 2006). For example, the transcriptional repressor Hes5, a downstream effector of the Notch pathway, negatively regulates oligodendrocyte differentiation, while we demonstrated in **Chapter 2** that inhibition of the expression of the Notch-ligand Jag1 by Foxp1 promotes neuronal differentiation (Kondo and Raff, 2000; Liu *et al*., 2006). In **Chapter 3** we evaluated the role of Sox4 in inhibiting oligodendrocyte differentiation of NSCs. Sox4 is a transcriptional activator and member of the SoxC family, sharing a high level of identity with the other SoxC factors Sox11 and Sox12 (Dy *et al*., 2008). It has been previously shown that Sox4, alone or together with other SoxC members, regulates neurogenesis and nervous system development in several contexts. Sox4 and Sox11 have been shown to promote neural tube development in chicken by directly inducing the expression of the pan-neuronal genes βIII-tubulin and MAP2 (Bergsland *et al*., 2006). Additionally, Sox4 and Sox11 have been suggested to promote neural progenitor proliferation and survival in mice, as well as promoting neurogenesis in the SGZ (Bhattaram *et al*., 2010; Mu *et al*., 2012). When depleting Sox4 in NSCs, we did not observe

reduced expression of the other SoxC transcription factors, Sox11 and Sox12. Therefore we have identified genes that are Sox4-dependent. However, this does not exclude that these genes might be additionally regulated by Sox11 and Sox12. Therefore it would be relevant to combine depletion of Sox4 with Sox11 and Sox12 knockdown in NSCs and then measure gene expression, to identify the genes that are shared targets amongst the SoxC factors.

In our study we identified multiple genes regulated by Sox4. Amongst the genes repressed by Sox4, we observed several positive regulators of oligodendrocyte differentiation. Accordingly, we identified genes positively associated with astrocyte and neuronal phenotypes as induced by Sox4. These observation are in agreement with the studies reporting Sox4 as promoting neuronal differentiation (Bergsland *et al*., 2006; Bhattaram *et al*., 2010; Mu *et al*., 2012). When comparing Sox4 targets across different cell types and species there is evidence of a context-dependency: Bergsland and colleagues report that in during neural tube development in chicken Sox4 induces expression of the pan-neuronal markers βIII-tubulin and MAP2, none of which could be identified as Sox4 targets in our datasets (Bergsland et al., 2006). Additionally, Tead2 and DCX expression have been described to be induced by Sox4 in mouse neural progenitors and adult mouse hippocampus respectively (Bhattaram et al., 2010; Mu *et al*., 2012). Supporting the Sox4 context-dependency hypothesis, neither of these genes can be identified as regulated by Sox4 in our datasets.

We demonstrate that Sox4 represses oligodendrocyte differentiation and that this is mediated by the direct activation of Hes5 expression by Sox4. Hes5, together with Hes1, has been suggested to induce NSC maintenance during mouse development (Ohtsuka *et al*., 2001). Hence it is possible that the repression of oligodendrogenesis mediated by Hes5 in NSCs is accompanied by the maintenance of neural progenitor traits. To this end it would be relevant to measure if Sox4 depletion, and therefore the inhibition of Hes5 expression, reduces the expression of NSC markers, such as Nestin or Sox2, during oligodendrocyte differentiation (De Filippis and Binda, 2012). Several stimuli induce oligodendrocyte differentiation *in vivo*, such as epidermal growth factor (EGF) and insulin growth factor-1 (IGF-1) (Patel and Klein, 2011). It would be interesting to investigate whether such stimuli could inhibit Sox4 expression as a mean to stimulate oligodendrogenesis. In our study we provide evidence of Sox4 repression of oligodendrocyte differentiation *in vitro.* However, these findings should be confirmed *in vivo*. To investigate whether Sox4 inhibits oligodendrocyte differentiation during cortical development in mice, one approach could consist in electroporating *in utero* Sox4-targeting shRNAs at E13.5 and analyze the number of oligodendrocyte progenitors 4 days later which are generated at the cortical ventricle (Langseth *et al*., 2010). Additionally, it would be relevant to investigate whether in these settings Sox4 depletion reduces Hes5 expression.

White matter injury (WMI) is a perinatal type of brain injury which is characterized by arrested maturation of oligodendrocyte development, ultimately resulting in myelination

failure in the developing white matter (van Tilborg *et al*., 2016). It would also be relevant to investigate whether Sox4 is increased in WMI-affected oligodendrocytes and thereby of Sox4 impair oligodendrocyte differentiation. To this end, it should be investigated whether mice which underwent WMI present higher levels of Sox4 in the oligodendrocyte progenitors when compared to uninjured littermates, and if this corresponds to increased Hes5 levels (van Tilborg *et al*., 2016).

In **Chapter 2** we describe Foxp1 as inducing neuronal differentiation of NSCs by repressing Jag1 expression, thereby negatively regulating Notch signaling. In **Chapter 3** we show that Sox4 induces the expression of the Notch downstream effector Hes5, thereby preventing oligodendrocyte differentiation of NSCs. Hence, Foxp1 and Sox4 have opposite effects in regulating the downstream signaling of the Notch pathway. Interestingly, upon Foxp1 depletion we observed decreased levels of Sox4 and Hes5 in our RNA-seq dataset (**Figure 2A and 2B**). It is possible that Foxp1, either directly or indirectly, positively regulates Sox4 expression. Supporting this, Sox4 expression has been found significantly reduced in the heart of Foxp1-/- mice (Wang *et al*., 2004). If confirmed in NSCs, Foxp1 might have a dual role in regulating Notch pathway: by repressing directly Jag1 expression and thereby promoting neuronal differentiation, and by inducing Sox4 expression, induce Hes5 expression and therefore prevent oligodendrocyte development. In conclusion, **Chapter 3** demonstrates that Sox4 is repressing oligodendrocyte differentiation by inducing the expression of Hes5 (see **Figure 3**). This study elucidates a novel mechanism that regulates oligodendrocyte differentiation and that could better our understanding of diseases which are characterized by impairment of oligodendrogenesis, such as WMI.

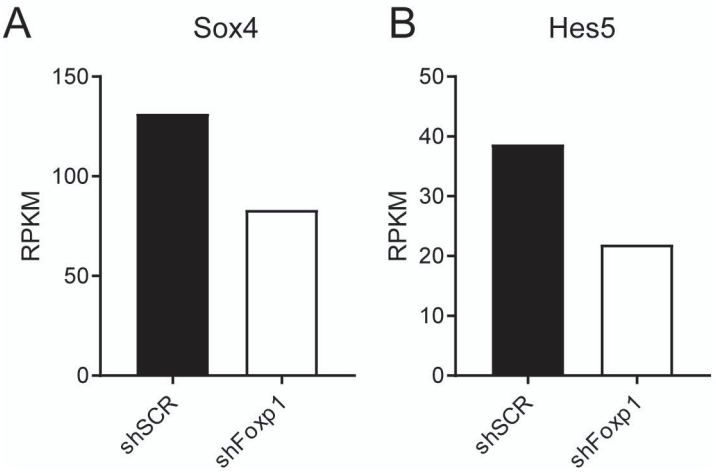

Figure 2: Sox4 and Hes5 expression is induced by Foxp1 in NSC.

Sox4 (**A**) and Hes5 (**B**) mRNA levels are reduced in the RNA-sequencing dataset upon Foxp1 KD in NSCs. RPKM: Reads per Kilobase per Million.

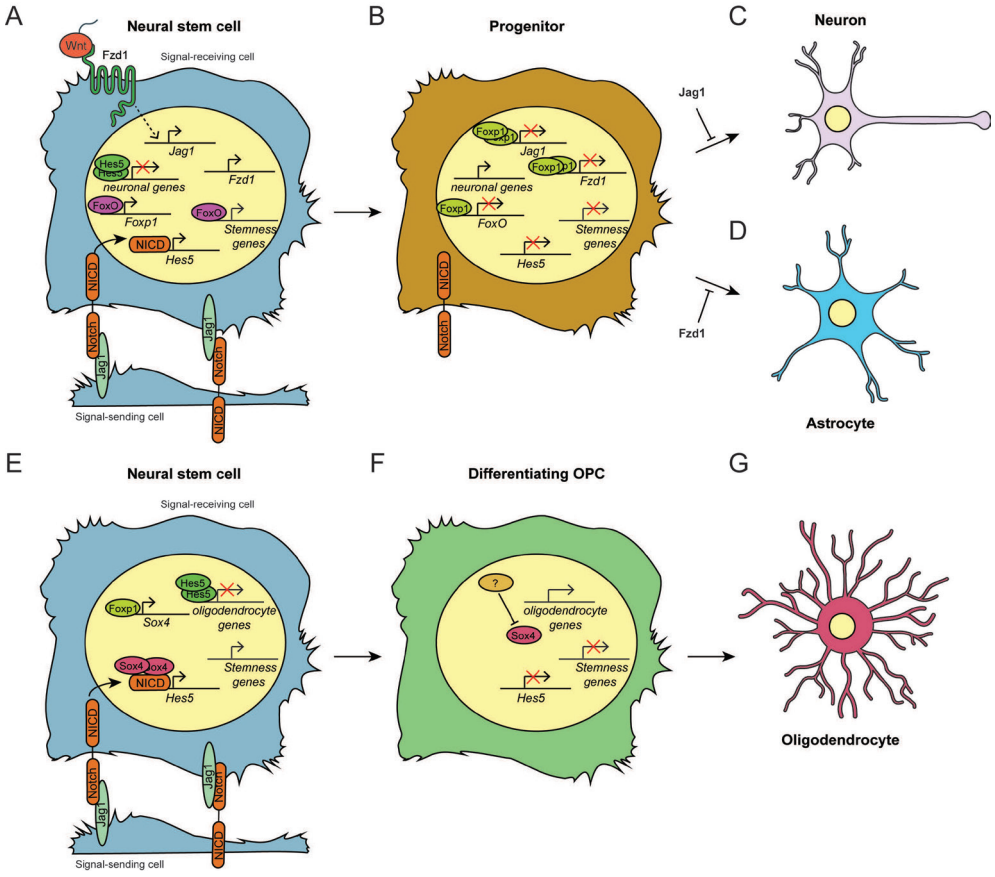

Figure 3: Schematic representation of Foxp1 and Sox4 working mechanism in NSCs.

In NSCs (**A**), Wnt binds Fzd1, inducing the expression of target genes such as *Jag1*. Binding of Jag1 to Notch results in NICD translocation to the nucleus, inducing *Hes5* expression. Hes5 represses the expression of pro-neuronal genes, contributing to NSC maintenance. FoxO induces both the expression of stemness genes and the expression of Foxp1. During differentiation (**B**) Foxp1 represses the expression of *FoxO*, resulting in decreased expression of stemness genes. Additionally, Foxp1 represses *Jag1* expression in the signal-sending cell, which results in reduced Notch activation and reduced *Hes5* levels in the signal receiving cell. (**C**) Repression of *Jag1* expression by Foxp1 promotes neuronal differentiation. (**D**) Repression of *Fzd1* by Foxp1 promotes astrocyte differentiation. In NSCs (**E**) Foxp1 induces *Sox4* expression. Sox4, together with NICD, increases expression of *Hes5*, which represses oligodendrocyte genes. In oligodendrocyte precursor cells (OPC) (**F**), Sox4 no longer induces *Hes5* expression, hence inducing expression of oligodendrocyte genes, which promote oligodendrocyte differentiation (**G**).

Neural stem cells to repair the hypoxic-ischemic brain

NSC transplantation is a promising tool to treat conditions affecting the central nervous system (Martino and Pluchino, 2006). Experimental studies in mice show how NSCs can improve motor function affected by Machado-Joseph disease, ameliorate cognitive deficits in a transgenic model of Alzheimer's disease, induce recovery upon neonatal excitotoxic brain injury and reduce symptoms in a model of multiple sclerosis (Blurton-Jones *et al.*, 2009; Mendonca *et al.*, 2015; Pluchino *et al.*, 2003; Titomanlio *et al.*, 2011).

Perinatal hypoxic-ischemic (HI) brain damage is a condition with high morbidity in the form of severe neurodevelopmental deficits in surviving infants (de Haan *et al*., 2006; Ferriero, 2004). Although upon HI endogenous NSCs respond to the insult by expanding in the SVZ, this response is limited by HI-induced apoptosis of the differentiating progeny deriving from NSCs, which fail to become functional neurons and glia (Brazel *et al*., 2004). Therefore a few studies have aimed at replenishing the endogenous NSCs by intracranial delivery of exogenous NSCs, or boost the differentiation capacity of endogenous NSCs by intranasal administration of mesenchymal stem cells (MSCs) (Daadi *et al*., 2010; Donega *et al*., 2013; van Velthoven *et al*., 2010b). Due to the limited therapeutic options available for HI brain damage (i.e. the only clinically applied current treatment is hypothermia), there is a need for effective ways to reduce the life-long consequences of this condition (Azzopardi *et al*., 2009; Gluckman *et al*., 2005). Preclinical studies that have demonstrated the benefits of NSC transplantation on reduction of tissue damage and functional impairment after HI, have administered the NSCs within 1-3 days upon HI (Ashwal *et al*., 2014; Daadi *et al*., 2010; Rosenblum *et al*., 2015). The study presented in **Chapter 4** aimed to investigate the effect of *late* allogenic NSC administration (i.e. at 10 days after insult) on HI brain injury. Previously, Daadi *et al.* elucidated the effect of intracranial NSC transplantation at 24 h after HI in 7-day-old rats (Daadi *et al*., 2010). In line with our findings, Daadi and colleagues reported improved motor performance of HI-affected animals after NSC treatment. Partially in contrast with our results, they showed that transplanted NSCs differentiated not only towards the neuronal lineage, but also towards the astrocyte lineage. This discrepancy can be explained by the different timing of transplantation, i.e. early versus late, in the study by Daadi *et al.* compared to our report. Supporting this hypothesis, Rosenblum and colleagues showed how NSCs transplanted in mice early (6-24 h) after adult stroke differentiated mostly into astrocytes, while delayed administration (7-14 d) yielded more neuronal progeny (Rosenblum *et al*., 2012). Additionally, another study showed how NSCs transplanted into the neonatal excitotoxic injured brain early after insult (4-72h) resulted into differentiation towards neurons, astrocytes and oligodendrocytes which eventually underwent apoptosis (Titomanlio *et al*., 2011). These observations indicate that the environment of the lesion changes over time and therefore influences the fate and survival of the transplanted cells. Previously it has been reported that transplanted NSCs differentiated into mature neurons several days after administration (Daadi *et al*., 2010; Park *et al*., 2002). In our study we did not address the long term fate of the transplanted NSCs, however our behavioral and histological results show that NSCs induced long-term functional recovery and tissue regeneration. We therefore suggest that the transplanted NSCs integrated in the host brain by becoming mature neurons. This is supported by the enhanced synaptic integrity observed after NSC transplantation, which might derive from NSC-derived neurons that grafted into the damaged hippocampus. Future studies should aim at verifying whether the transplanted NSCs-derived neurons are replacing the damaged synapses or if these synapses derive from endogenous progenitors. For instance, genetic labeling of NSCs could be used to verify the

long-term integration of the progeny of the transplanted NSCs into the hippocampus after HI. If the transplanted NSCs elicit the differentiation of endogenous progenitors in the HI brain, this could be advantageous over the long-term integration of the transplanted NSCs, as it would avoid any kind of host-graft immune reaction. Moreover, exogenous NSCs could be engineered to improve their capacity to engraft, to boost the endogenous progenitor response, or to reduce inflammation after HI. NSCs could for instance be modified to express brain derived neurotrophic factor (BDNF) or sonic hedgehog (SHH), two secreted factors known for their pro-neurogenic effect (Lai *et al*., 2003; Park and Poo, 2013; Sims *et al*., 2009). Moreover, in order to improve their anti-inflammatory properties, NSCs could be modified to overexpress cytokines like interleukin-10 (IL-10), since it has been shown that overexpressing IL-10 in transplanted NSCs boosts their immuno-suppressive capacity in a mouse model of experimental autoimmune encephalitis (Yang *et al*., 2009). Modifications of stem cells by overexpressing certain factors is also discussed below in this Chapter for MSCs.

Our results indicate that NSCs are capable of migrating towards the lesion area when transplanted into the contralateral hemisphere. This phenomenon could be elicited by the secretion of chemoattractants within the damaged tissue: previous reports showed how NSCs can respond to soluble factors like stromal cell-derived factor 1α (SDF-1α) by migrating towards the injured areas (Imitola *et al*., 2004). This migratory capacity of NSCs might be of great importance as it would allow NSCs to be administrated by less invasive, non-local routes including intravenous (i.v.) or intranasal (i.n.) application. Both i.v. and i.n. administration of NSCs have been tested already, showing that NSCs are capable to reach the brain by these routes (Einstein *et al*., 2007; Lee *et al*., 2005; Reitz *et al*., 2012; Wu *et al*., 2013), therefore it would be relevant to study whether i.v. and i.n. administration of NSCs is beneficial for neonatal HI. Being much less invasive than intracranial delivery, i.v. and i.n. administration of NSCs could be repeated multiple times after HI, potentially increasing their therapeutic effects.

Transplanted NSCs can either directly replace the damaged tissue or indirectly dampen tissue damage by secreting soluble factors. The latter explanation is supported by the inhibition of microglial activation mediated by NSC's secretome shown in our study, which indicates that NSCs can reduce neuroinflammation, probably by suppressing the secretion of pro-inflammatory cytokines by immune cells, such as TNF-α (Lee *et al*., 2008). Furthermore, NSCs might secrete other cytokines that attract neural progenitors or induce their survival and differentiation therefore boosting the endogenous regenerative response. To this end, it would be relevant to identify cytokines that are secreted in NSCs upon exposure to HI brain extracts utilizing for instance a luminex multiplex assay. Complementary, one could identify the genes that are higher expressed in NSCs upon stimulation with HI brain extracts by utilizing RNA-seq. We observed that transplanted NSCs localized in the lesion site at the hippocampus and were surrounded by endogenous astrocytes and progenitor

cells to form a neurogenic niche. It is likely that transplanted NSCs might interact with these endogenous cells both through their secretome as well as cell-to-cell contact, to guide the regenerative response.

Previously our group demonstrated the efficacy of intranasal MSC transplantation in inducing repair of HI brain injury through the support of proliferation and differentiation of endogenous stem cells into differentiated neurons and oligodendryocytes (Donega et al., 2014; van Velthoven *et al*., 2010c) A possible advantage of NSCs over MSCs is that NSC transplantation provides a source of already specified neural progenitors that are ready to replace the largely damaged tissue upon HI, whereas MSCs rely on inducing endogenous progenitor differentiation. These endogenous progenitors might also be partially depleted upon HI which might dampen the response to MSC treatment upon HI (Donega *et al*., 2014; van Velthoven *et al*., 2010c). It is therefore possible that NSCs might prove to be more effective than MSCs in case of more severe HI lesions. To this end, it would be relevant to compare NSC and MSC treatment side-by-side upon HI brain injury, in order to verify which of the two approaches is more effective in restoring the damaged brain relative to brain damage severity. Moreover, further studies should elucidate the possible advantage of multiple NSC gifts via less invasive routes (i.v. or i.n). In contrast, a possible disadvantage of NSCs over MSCs is that NSCs are much more difficult to isolate and expand than MSCs: MSCs can be easily isolated from bone marrow or adipose tissue of the patient (Hass *et al*., 2011) whereas NSCs can be isolated from post-mortem tissues (limiting their availability) or generated from induced pluripotent stem cells (iPSC) (thus carrying the inherent risk of tumorigenesis). In our study we propose the use of embryonic NSCs, which collection is more difficult due to ethical issues. Therefore further studies should focus on different sources of NSCs to bring this powerful tool closer to a possible clinical application.

Genetically modified mesenchymal stem cells to boost HI treatment

Our group and others have shown that MSC transplantation reduces lesion size and improves functional outcome in preclinical animal models of ischemic brain insults (Chen *et al*., 2001; Lee *et al*., 2010; van Velthoven *et al*., 2010a, c; van Velthoven *et al*., 2012). One of the largest differences with NSCs is that MSCs do not survive longer than a few days after transplantation; only 1% of transplanted MSCs survive in the brain when measured at 18 days after transplantation (van Velthoven *et al*., 2011). So whereas NSCs can integrate in the brain and replace tissue, the current opinion on MSCs is that, rather than differentiating into neurons or glia themselves, MSCs *promote* endogenous regeneration of the damaged tissue by releasing soluble factors that induce angiogenesis, neurogenesis and formation of synapses (van Velthoven *et al*., 2011; van Velthoven *et al*., 2012). Our hypothesis that the damaged brain milieu regulates the MSC's secretome was confirmed by our experiments in which MSCs, when cultured *in vitro* with ischemic brain extracts, expressed a differential

plethora of cytokines and growth factors in comparison to MSCs cultured with control brain extracts (Chen *et al*., 2002; Qu *et al*., 2007; van Velthoven *et al*., 2010c, 2011)z. Taken together, these observations suggest that MSC-induced secretion of certain trophic factors plays a prominent role in repairing the ischemic brain. In **Chapter 5**, we aimed to investigate whether the regenerative potential of MSCs to treat HI could be improved by modifying MSCs to overexpress trophic factors. Effects of overexpressing MSCs were tested both on NSC differentiation *in vitro* and upon HI brain damage *in vivo*. The 4 neurotrophic factors used for overexpression were chosen on their well-known capacity to promote neurogenesis or neuroprotection and will be discussed shortly below. Brain derived neurotrophic factor (BDNF) is well-known for its neurotrophic characteristics, and plays an important role in angiogenesis, neurogenesis and synaptogenesis (Park and Poo, 2013; Schabitz *et al*., 2007). Moreover, BDNF administration has been shown to ameliorate the effects of adult cerebral ischemia (Schabitz *et al*., 2007). Epidermal growth factor-like 7 (EGFL-7) is a secreted protein known as a negative regulator of Notch signaling (Dikic and Schmidt, 2010). Inhibition of Notch through EGFL-7 can possibly induce neuronal differentiation. Persephin (PSP) is a ligand from the TGFβ family which has been demonstrated to induce neuroprotection (Milbrandt *et al*., 1998; Tomac *et al*., 2002). Sonic hedgehog (SHH) is known to drive migration and differentiation of neural progenitors towards the neuronal and oligodendrocyte lineage (Lai *et al*., 2003; Sims *et al*., 2009). Our results in **Chapter 5** indicate that all modified MSCs were able to boost NSC differentiation *in vitro* in comparison to control MSCs. In line with our study, it has been previously shown that BDNF induces proliferation of E13-derived NSCs (Barnabe-Heider and Miller, 2003; Islam *et al*., 2009). While SHH has been demonstrated to induce proliferation of E15 neural progenitors and stimulate their differentiation towards all three lineages, we only observed increased *neuronal* differentiation of NSCs co-cultured with SHH-overexpressing MSCs (Palma and Ruiz i Altaba, 2004). EGFL7, through negative regulation of Notch signalling, has been demonstrated to inhibit proliferation and self-renewal of NSCs *in vitro* (Schmidt *et al*., 2009). In contrast, we observed that EGFL7-overexpressing MSCs increased proliferation of NSCs *in vitro* (Schmidt *et al*., 2009). This discrepancy could be attributed to a different effect of EGFL7 on embryonic versus adult neural progenitors. Furthermore we investigated the effect of the modified MSCs *in vivo* upon HI. Previous studies indicate that treatment with exogenous BDNF reduced infarct size upon experimental stroke, which aligns with our observations that BDNF-overexpressing MSCs improved motor function and reduced lesion size upon neonatal HI (Jiang *et al*., 2011; Ploughman *et al*., 2009; Schabitz *et al*., 2007). Accordingly, our group has previously shown that BDNF-overexpressing MSCs have a beneficial effect on neonatal stroke as well (van Velthoven *et al*., 2013). Our results indicate that MSC that secrete EGFL7 are as effective as BDNF-overexpressing MSCs in improving motor performance; this however was not accompanied by a reduced lesion size or augmented proliferation. Given the absence of an effect of EGFL7-secreting MSCs on lesion area when compared to control MSCs, it is possible that EGFL7-MSCs influence the remodeling of the corticospinal tract (CST) rather than the

brain lesion, thus explaining the effect on motor performance. In fact EGFL7, via inhibition of the Notch pathway, could guide the rerouting of axons of the motor neurons throught the CST, as modulation of Notch signalling has been shown to regulate axon guidance (Giniger, 2012). Overexpression of PSP and SHH in MSCs did not show any beneficial effect upon HI, and rather a negative effect in case of SHH. This could be explained by the fact that these neurotrophic factors need to be expressed in a temporal and spatial restricted manner in order to exert their function. Overexpression of factors in transplanted MSCs is a quite coarse approach that does not allow the 'tailor-made' fine tuning that occurs under physiological conditions.

In conclusion, **Chapter 5** shows how the intranasal administration of MSCs supra-secreting growth factors can be exploited to modulate the therapeutic potential of stem cells upon HI. However, the 'ideal' factors have to be chosen to induce favorable events, such as neuronal and oligodendrocyte differentiation at the right time, while limiting possible detrimental effects for HI recovery, such as gliosis. Additionally, it has to be taken into account that the overexpressed factors might not only influence the desired cell target, but also the MSCs themselves or other cell types such as microglia. Finally, it emerges from our observations that tweaking only one factor may not be the right answer to improve the efficacy of stem cells. Furthermore, transplanted MSCs have been shown to exert their function by responding to the environmental cues induced by HI (van Velthoven *et al*., 2010c, 2011). Hence, preconditioning of MSCs in a 'soup' of HI-brain extracts prior to transplantation might be a better solution to boost the regenerative potential of MSCs upon the actual physiological cues.

Exosomes: an alternative to cell-based approaches?

Exosomes are a subtype of secreted extracellular vesicles that play a fundamental role in cell-cell signaling. In **Chapter 6** we speculate on the potential of using exosomes for treating CNS conditions. Our group also explored the potential of MSC-derived exosomes (MSC-exo) to treat HI brain damage. Hence we administered exosomes that were derived from human embryonic stem cell-derived MSCs via the intranasal route to HI mice at 10 days after the insult. After measuring motor function and lesion area of the treated animals, we came to the conclusion that in our hands treatment with MSC-exo had no beneficial effect compared to vehicle treatment. On the contrary, a trend indicates that the high dose of MSC-exo worsened both the motor performance and the neuronal damage after HI (**Figure 4A and 4B**). This could be explained by the applied dose of exosomes which might have been too low to induce positive effects, by the human source of the exosomes which might carry inherent cross-species immune reactivity, or by the intranasal administration route which could have prevented the exosomes from reaching the damaged area. In the future it would be relevant to label the exosomes with a fluorescent lipophilic dye, such as PKH26, in order to visualize whether the exosomes reach the target area. In contrast to our findings, a study

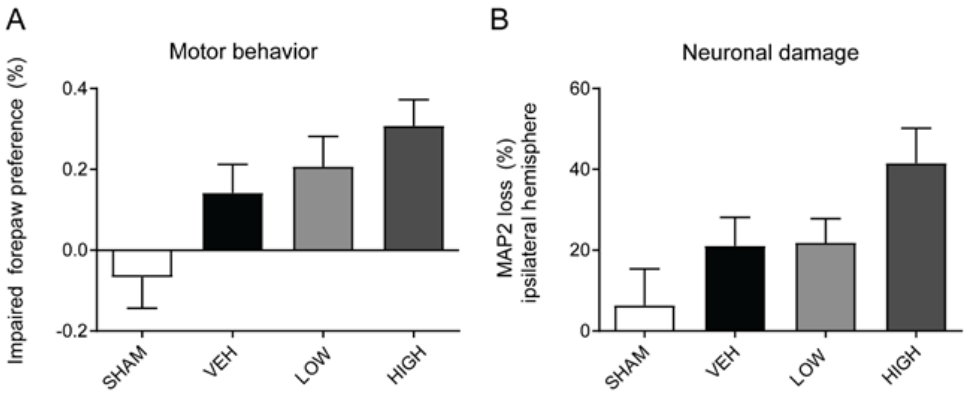

Figure 4: Effect of MSC-derived exosomes on motor behavior and neuronal damage after HI.

Neonatal P9 mice subjected to HI received intranasal administration of 0.25 µg (LOW) or 0.5 µg (HIGH) exosomes at 10 days post-HI. (**A**) Cylinder rearing test was performed at 18 days after intranasal treatment to measure motor performance. (**B**) Analysis of neuronal damage was measured as MAP2-negative area in the ipsilateral hemisphere versus the contralateral hemisphere at 18 days upon treatment. SHAM: sham-treated animals, VEH: intranasally PBS-treated HI animals.

from Xin and colleagues showed how i.v. administration of 100 ug of MSC-exo induced functional recovery and neurovascular regeneration in stroke-affected rats (Xin *et al*., 2013). Similarly, another study showed how i.v. treatment with 100 ug of MSC-exo reduced lesion size and preserved neurological function in rats upon ischemic stroke (Chen *et al*., 2016). These differences with our results could be explained both by the (~5-10x) higher dose and the i.v. route utilized in these studies. Additionally, Drommerschmidt and colleagues demonstrated that MSC-derived extracellular vesicles (EV) (which include exosomes) were effective in treating inflammation-induced preterm brain injury in rats. Drommerschmidt *et al.* described how the intraperitoneal treatment with two repetitive doses of 1×108 cell equivalents of MSC-EVs per kg bodyweight ameliorated neuronal degeneration, reduced microglia activity and reactive astrogliosis. This effect of the MSC-EV was accompanied by a reduction of the myelination deficits and white matter abnormalities (Drommelschmidt *et al*., 2017). In this case, the use of the whole EV bulk population might even be advantageous as it can include more beneficial factors than just a subpopulation of EVs such as exosomes. Similarly, the potential of MSC-derived vesicles has also been shown in an ovine model of preterm brain injury: fetuses exposed to HI that were treated *in utero* intravenously with 1 dose of 4×107 cell equivalents of MSC-EV, reported improved brain function including reduced HI-induced seizures (Ophelders *et al*., 2016). Taken together these studies demonstrate how MSC-exo/-EV can potentially be applied for treating conditions affecting the newborn brain, including HI. While further studies are needed to explore the clinical efficacy and safety of these vesicles, still doubts remain as whether EV/exosomes treatment should be preferable to MSC-/NSC-based therapies for HI brain injury. On one hand EV/exosomes have the advantage of being an "off-the-shelf", ready to use drug, with low immunoreactivity

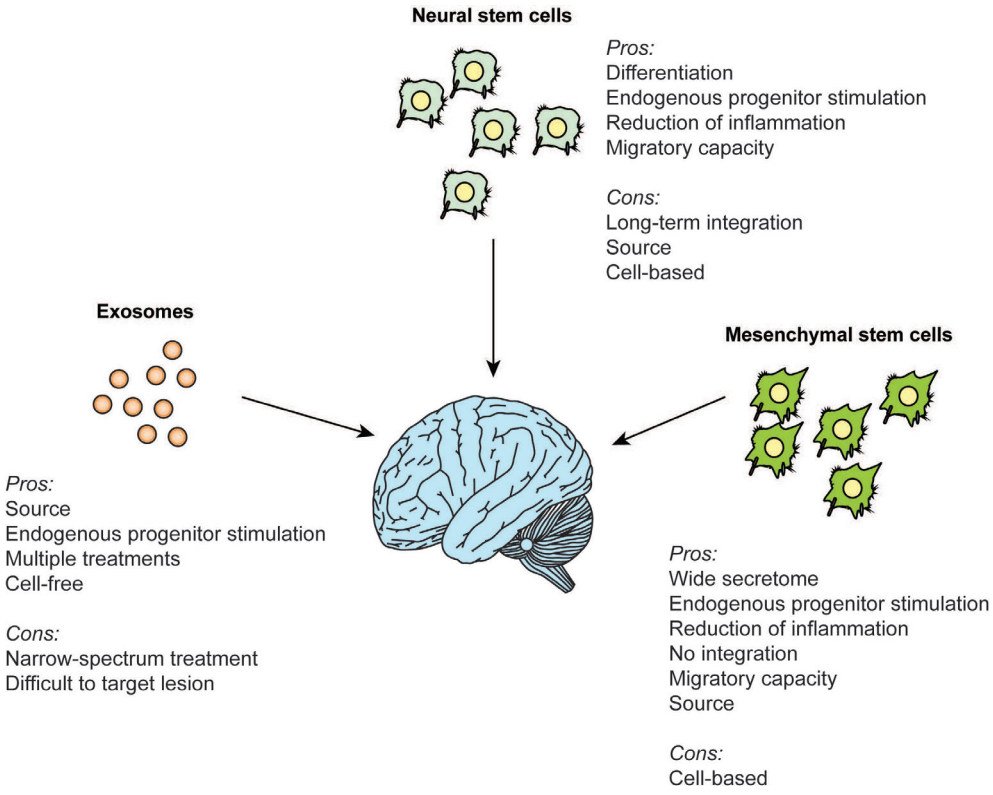

Figure 5: Therapeutic strategies for HI brain injury.

Schematic representation indicating the pros and cons of NSC, MSC or exosome treatment for HI brain injury.

and virtually *non*-tumorigenic properties. On the other hand, MSCs/NSCs have the capacity to respond to the environment, reach the damaged area as they can migrate in response to chemokines, and they will provide a wider array of stimuli. These include not only EVs/exosomes, but also growth factors, cytokines and cell-to-cell contact. In conclusion, only a side-by-side comparison of EVs/exosomes and cell-based therapies can provide insights on the most efficacious and safest treatment for HI brain injury (see **Figure 5**).

In conclusion the findings reported in this thesis have provided novel mechanistic insights into the regulation of NSC differentiation by the transcription factors Sox4 and Foxp1. Moreover we provided evidence that NSC transplantation is a valid and promising approach to treat HI brain injury. Additionally, we described how MSCs can be modified to over-express secreted factors that promote lesion regeneration and functional recovery upon HI. Finally we speculated on the use of exosomes as an alternative to cell-based therapies to treat CNS-related conditions.

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APPENDICES

Summary in English

Neurogenesis and gliogenesis are processes that occur during development of the CNS as well as after insult of the nervous system. These tightly regulated processes take place in specialized niches during embryogenesis and adulthood to generate functional cells, starting from defined progenitor cells such as neural stem cells (NSCs). Here we took a multifaceted experimental approach to better understand NSC biology both in vitro and in vivo. Transcriptional regulation is essential since the expression of specific genes is the key to control timing and fate of the differentiation process.

In this thesis two NSC transcriptional regulators, Foxp1 and Sox4 are shown to be crucial for control of neurogenesis and gliogenesis respectively. Mutations in the Foxp1 gene have been associated with speech defects, autism and other intellectual disabilities, as well as being defined as necessary for neurogenesis. In the work presented in this thesis, we sought to define the molecular mechanisms mediated by Foxp1 that regulate NSC differentiation. Sox4 has been described as inhibitor of gliogenesis and myelination in oligodendrocyte precursor cells. In this context we investigated the role and molecular mechanisms underlying Sox4-mediated regulation of oligodendrogenesis.

The use of cellular strategies to repair CNS insults has been widely investigated in recent years. Here we further evaluate the therapeutic potential of NSCs and MSCs to treat perinatal hypoxicischemic brain damage (HI). Moreover, the hypothesis of the use of exosomes as a cell-free alternative as a therapeutic option upon brain insults and HI is here examined.

Nederlandse samenvatting

Neurogenese en gliogenese zijn processen in het centraal zenuwstelsel (CZS) die zowel tijdens de ontwikkeling als na schade optreden. Deze strikt gereguleerde processen vinden, zowel gedurende embryogenese als op volwassen leeftijd, plaats in gespecialiseerde 'niches' van het CZS om nieuwe functionele cellen te verkrijgen vanuit gedefinieerde voorlopercellen zoals neurale stamcellen (NSCs). In dit proefschrift hebben we een meerzijdige experimentele aanpak gebruikt om de biologie van NSCs in vitro en in vivo beter te begrijpen.

Transcriptionele regulatie is cruciaal omdat de expressie van specifieke genen in NSCs essentieel is om het tijdstip en de uitkomst van het differentiatie proces te bepalen. In dit proefschrift tonen we aan dat twee transcriptiefactoren, Foxp1 en Sox4, essentieel zijn voor het aansturen van respectievelijk neurogenese en gliogenese. Mutaties in het Foxp1 gen zijn geassocieerd met spraakafwijkingen, autisme en andere verstandelijke beperkingen. In dit proefschrift hebben we uitgezocht welke moleculaire mechanismen, aangestuurd door Foxp1, betrokken zijn bij differentiatie van NSCs. Sox4 is eerder beschreven als een remmer van gliogenese en myelinisatie in oligodendrocyt voorlopercellen. Naar aanleiding daarvan hebben we verder onderzocht wat de rol en onderliggende moleculaire mechanismen zijn van Sox4 tijdens de regulatie van oligodendrogenese.

In de afgelopen jaren is er veel onderzoek gedaan naar cel-therapie om het beschadigde CZS te repareren. We hebben in dit proefschrift onderzoek gedaan naar de mogelijkheid om met neurale en mesenchymale stamcellen perinatale hypoxisch-ischemische (HI) hersenschade te herstellen. Verder hebben we ook het gebruik van exosomen, een cel-vrije therapie, als een alternatieve optie voor HI hersenschade onderzocht.

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Curriculum Vitae

Luca Vittorio Braccioli was born on October 20th in Milan, Italy. After completing his secondary education at the lyceum "Alessandro Volta" in Milan, in 2005 he started studying Molecular Biotechnology at the University of Milano-Bicocca in Milan, where he obtained a Bachelor's degree in 2008. In 2011 he obtained his Master's degree in Industrial Biotechnology at the University of Milano-Bicocca in Milan. He performed his Master's research project on microRNAs and breast cancer under the supervision of Marilena Iorio at the National Cancer Institute in Milan, where he spent 8 months working as research assistant after graduating. In 2011 Luca moved to the Netherlands to begin his PhD under the supervision of Dr. Paul Coffer, Dr. Cobi Heijnen and Dr. Cora Nijboer at the University Medical Center, Utrecht. The results of his PhD are presented in this thesis.