Reprogramming of stem cells in the peripheral nervous system to CNS stem cells

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Dekanin: Prof. Dr. Meike Piepenbring

Gutachter: Prof. Dr. Hermann Rohrer (Erstgutachter)
Prof. Dr. Bernd Grünewald (Zweitgutachter)

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„Das Schönste, was wir entdecken können, ist das Geheimnisvolle.“

Albert Einstein

Abbreviations

A	Adenosine
Abcc9	ATP-binding cassette, sub-family C
Acan	Aggrecan
Acta2	Actin, alpha 2, smooth muscle, aorta
ALS	Amyotrophic lateral sclerosis
AP	Anteroposterior
BDNF	Brain-derived neurotrophic factor
Bgn	Biglycan
BMP	Bone morphogenetic protein
Bp	Base pairs
Brn4	POU domain, class 3, transcription factor 4
BSA	Bovine serum albumin
°C	Grad Celsius
cAMP	3‘-5‘-cyclic adenosine
CC	Corpus callosum
CC1	Crenarchaeal chromatin protein 1
cDNA	Complementary DNA
CD	Cluster of differentiation
CEE	Chickem embryo extract
CNP, CNPase	2‘,3‘-Cyclic-nucleotide 3‘-phosphodiesterase
CNS	Central nervous system
Col2a1	Collagen, type II, alpha 1
Col9a1	Collagen, type IX, alpha 1
DAPI	4‘, 6‘-diamidino-2-phenylindole dihydrochloride
Cx	Cortex
div	Days in vitro
dg	Dentate gyrus
Dlx	Distal-less homeobox
DMEM	Dulbecco‘s Modified Eagle‘s medium
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
DRG	Dorsal root ganglion
E	Embryonic day
EDTA	Ethylendiamintetraacetat
Efcab7	EF-hand calcium binding domain 7
EGF	Epidermal Growth Factor
Emx1	Empty spiracles homeobox 1
Enpep	Glutamyl aminopeptidase
ESCs	Embryonic stem cells
et al.	et alia (stands for: and others)
Fabp7	Fatty acid binding protein 7, brain
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
Fmod	Fibromodulin
Foxg1	Forkhead box G1
FP	Floor plate

Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gbx2	Gastrulation brain homeobox 2
GCL	Granule cell layer
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GO	Gene Ontology
GNP	Granule neuron progenitor
H	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hox	Homeobox
Id	inhibitor of differentiation (Id) family
iPSCs	Induced pluripotent stem cells
IPTG	Isopropyl-β-D-thiogalactopyranosid
Irx3	Iroquois-class homeodomain protein Irx3
ITRs	Inverted terminal repeat sequences
Kcnj8	Potassium channel, inwardly rectifying subfamily J, member 8
Klf4	Kruppel-like factor 4
Krox20	Early growth response 2 (Egr2)
LacZ	Beta-D-galactosidase
Lgals3	Lectin, galactoside-binding, soluble, 3
Ly6a	Lymphocyte antigen 6 complex, locus A
m	Meter
mA	Milli amper
MAG	Myelin associated glycoprotein
MAPk	Mitogen-activated protein kinase
MBP	Myelin basic protein
M	Molar
MEM	Minimum Essential Media
Mgp	Matrix Gla protein
mg	Milli gramm
µg	Micro gramm
min	Minutes
ml	Milli liter
µl	Micro liter
mm	Mus musculus
µm	Micro meter
MOG	Myelin Oligodendrocyte Glycoprotein
mPB	mPbase
MSCs	Mesenchymal stem cells
mRNA	Messenger RNA
Myc	Myelocytomatosis oncogene
Nanog	Nanog homeobox
n	Number
Ncad	Cadherin-N
N-CAM	Neural Cell Adhesion Molecule
NCSCs	Neural crest stem cells
NC	Neural crest

NGF	Nerve growth factor
Ng2	Neuron-glial antigen 2
Ngn2	Neurogenin
Nkx	Nkx-homeodomain factors
nm	Nano meter
Notch1	Notch homolog 1, translocation-associated (Drosophila)
Nr2e1	Nuclear receptor subfamily 2, group E, member 1
NRG1	Neuregulin 1
NS	Neurosphere
NSC	Neural stem cell
NT3	Neurotrophin 3
Ω	Ohm
OB	Olfactory bulb
Oct	Major octamer-binding protein
Ogn	Osteoglycin
Olig	Oligodendrocyte transcription factor
OPC	Oligodendrocyte Precursor Cells
OSKM	Oct4, Sox2, Klf4, c-Myc
OSK	Oct4, Sox2, Klf4
P#	Passage#
P	Postnatal day
Pax	Paired box protein
PB	Piggy Bac
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
Pdgfr	Platelet-derived growth factor receptors
Phox2b	Paired-like homeobox 2b
P0	Protein zero
p75	p75 Low-Affinity Nerve Growth Factor Receptor
PLP	Proteolipid-Protein
pMN	Motoneuron domain
PMP-22	Peripheral myelin protein 22
pNCSCs	Palatal neural crest-derived stem cells
PNS	Peripheral Nervous System
POA antigen	Proligodendroblast antigen
P/S	Penicillin/Streptomycin
Ptprz1	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RF	Roof plate
RGP	Radial glia progenitor
Rgs5	Regulator of G-protein signaling 5
rNCSCs	Reprogrammed neural crest-derived stem cells
RT-PCR	Real-time polymerase chain reaction
S	Sympathetic ganglia
S1pr3	Sphingosine-1-phosphate receptor 3
SC	Spinal cord

SCSCs	Spinal cord stem cells
SEM	Standard error of the mean
SGZ	Subgranular Zone
Shh	Sonic hedgehog
S100	S100 calcium binding protein
Slc1a3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3
Slug	Zinc finger protein SLUG
Smad	SMA/MAD related; small body size/mother against dpp related
Snai2	Snail family zinc finger 2
Sox	Sry-box containing homeodomain
Sry	Sex determining region Y
Spp1	Secreted phosphoprotein 1
SVZ	Subventricular zone
T	Thymidine
TAE	TRIS-Acetat-EDTA
Taq	Thermus aquaticus
Tcfap2	Transcription factor Activating Protein 2
TE	TRIS-EDTA
TGF	Transforming Growth Factor
TH	Tyrosine hydroxylase
Timp2	TIMP metallopeptidase inhibitor 2
Tnc1	Tenascin 1
trk	Neurotrophic tyrosine kinase receptor
Tuj1	Neuron-specific class III beta-tubulin
Twist	Basic helix-loop-helix transcription factor Twist
v/v	Volume/Volume
V	Volt
w/v	Weight/Volume
x-Gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranoside
Zfp	Zinc finger transcription factors
Zic1	Zinc finger protein of the cerebellum 1

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1. Introduction

1.1 The development of vertebrate embryos

The basic anatomical development of vertebrates is comparable in almost all species. After fertilisation, the zygote is formed, division continues and the blastocyst is generated. The blastocyst is built of an epithelial cell layer with inner hollow space. The epithelial layer develops to the three germ layers called entoderm, mesoderm and ectoderm. The nervous system and epidermis are derived from the ectoderm. The entoderm gives rise to the gut and its adnexa. Muscles, connective tissues and other parts of the body are derived from the mesoderm (Alberts, 2009).

1.2 The development of the vertebrate nervous system

The nervous system is split into the central nervous system (CNS), including the brain and spinal cord, and the peripheral nervous system (PNS) that contains the autonomic, peripheral sensory and the enteric nervous system. Both are derived from the ectoderm but their development and generated PNS and CNS derivatives are completely different as will be described below.

The peripheral nervous system is derived from the neural crest, a transient structure, sometimes called the fourth germ layer that originates from the ectoderm. The bona fide neural crest is located between the future neural and non-neural ectoderm and is called neural plate border at this stage. The neural crest is induced through a regulatory network including BMP, Wnt and FGF, which are secreted the from neural plate, underlying mesoderm and adjacent non-neural ectoderm (Figure 1A). Intermediate levels of BMPs induce the expression of „neural crest specifiers“ such as Snail, Slug, Tcfap2, Sox9 and FoxD3 (Figure 1A) (reviewed in Meulemans and Bronner-Fraser 2004). High BMP signaling leads to the induction of the epidermis. Low BMP signaling, resulting from the presence of BMP inhibitors, such as noggin, chordin and follistatin

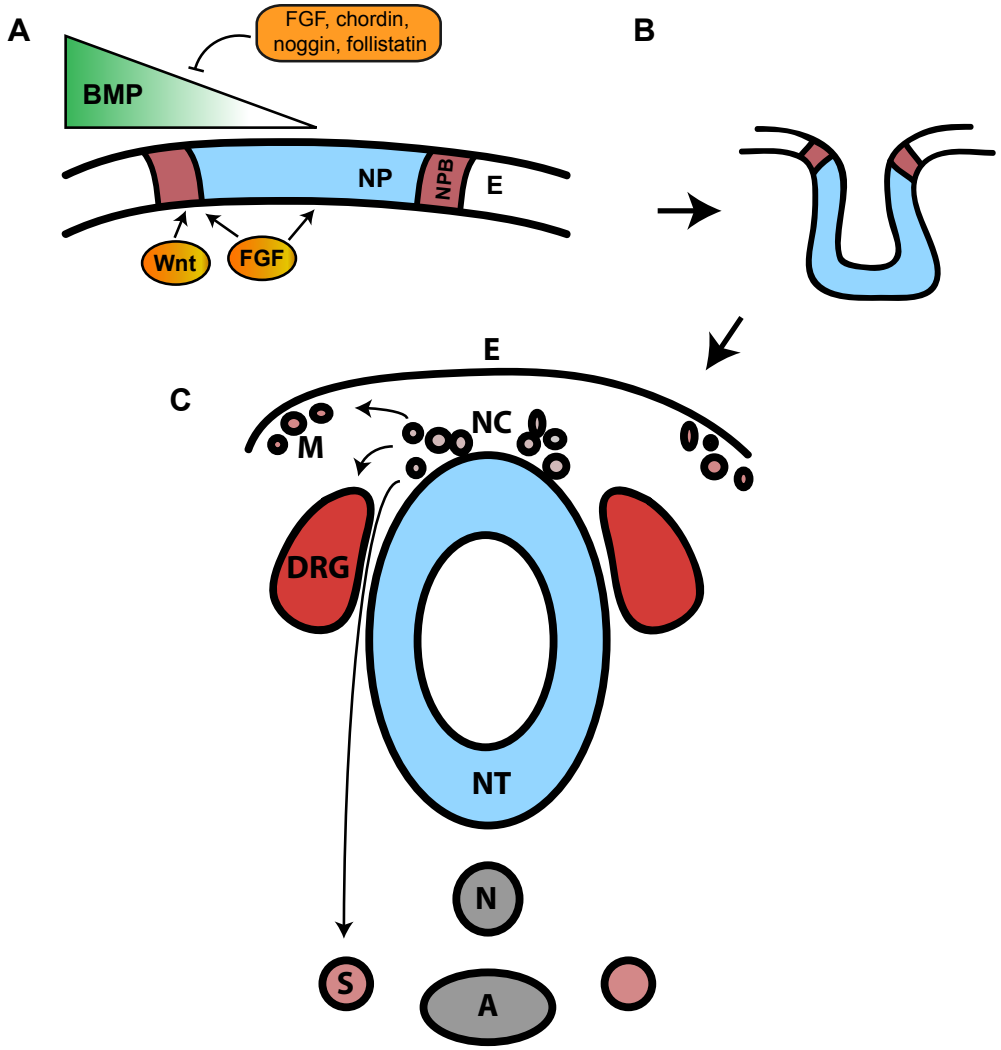

Figure 1: The development of the nervous system. (A) displays the generation of the neural crest (NC) controlled by BMP, Wnt and FGF and the BMP antagonists such as FGF, chordin, noggin and follistatin. After neurulation (B), NC cells start to migrate to their destinations. (C) At the trunk region they differentiate into melanocytes (M), generate the DRG and the sympathetic ganglia (S). Neural plate (NP), neural plate border (NPB), epidermis (E), notochord (N), dorsal aorta (A), neural tube (NT).

(Figure1A) (LaBonne and Bronner-Fraser, 1998), and FGF signaling induce the neural plate that will form the entire CNS later in development (Rentzsch et al., 2004; Londin et a., 2005). Wnt signaling also plays a role in neural crest induction and it is thought that FGF is one of the inducers of Wnt. In the chicken, it was shown that Wnt inhibition results in the failure of neural crest formation (Patthey et al., 2009). In the mouse, Wnts are expressed just before the neural crest cells start to migrate and Wnt inhibition results in abnormalities of neural crest derivatives. Wnt signaling seems not be essential

in the early development of the mouse neural crest (Ikeya et al., 1997). However, FGF does not only induce Wnt signaling. When FGF receptors are blocked in ectoderm, the neural crest fails to develop (reviewed in Milet and Monsoro-Burq, 2012). This is explained by the contribution of FGF signaling to the adjustment of the intermediate level of BMP signaling by inducing an inhibitory phosphorylation on the Linker domain of Smad1 (Streit et al., 2000; Kudoh et al., 2004). The regulatory network of neural crest inducer and induced genes is very complex and not fully understood yet.

Neurulation starts after the induction of the neural plate, neural plate border and epidermis. During this process the neuroepithelium, including the neural plate and neural plate border, begins to form neural folds. The neural crest remains located in a dorsolateral position during this process (Figure 1B) (Murphy and Bartlett, 1993). Neural folds fuse to each other at the midline to generate the neural tube which will form the brain and spinal cord later in development. In the mouse, the closure of the neural tube begins at E8 (Copp et al., 2003).

1.2.1 The development of the peripheral nervous system (PNS)

The peripheral nervous system (PNS) is derived from the neural crest, which is built of multipotent stem cells (Le Douarin, 1982; Le Douarin and Kalcheim,1999). The neural crest cells delaminate from the closing neural tube after epithelial- mesenchymal transition and migrate through the embryo until they reach their destinations and differentiate into neurons, melanocytes, endocrine and glial cells (Le Douarin, 1982; Le Douarin and Kalcheim,1999). The first population of neural crest cells starts to migrate at the cranial region, where they generate the cranial sensory ganglia, parasympathetic ganglia and in addition mesenchymal derivatives that will form the skeleton of the face and other cranial mesenchymal derivatives (Le Douarin and Kalcheim, 1999). Vagal (somites 1 to 7) the neural crest cells form the enteric nervous system. Trunk neural crest cells migrate ventrally through the rostral part of the somites and at their final destinations they generate the dorsal root ganglia (DRG), sympathetic ganglia (S)

(Figure 1C) and chromaffin cells of the adrenal gland. Melanocytes are generated from neural crest cells at all axial levels migrating beneath the ectoderm (Squire, 2008).

1.2.2 Dorsal root ganglia (DRG)

The DRG contains cell bodies of the neurons that transmit information from sensory receptors in the periphery into the spinal cord. While early migrating neural crest cells generate the sympathetic and enteric nervous system, the glial cells and neurons from the DRG are generated by later migrating neural crest cells (Lefcort, 2007). The DRG is built up by three cell migrating waves. The first wave of migrating neural crest cells forms the ventrolateral neurons of the DRG (Wegner and Stolt, 2005; Wakamutsu et al., 2000; Marmigiere and Ernfors, 2006; Bononi and Bradley, 2006). This population comprises proprioceptive and mechanoreceptive neurons, which express TrkC and TrkB (Carr et al., 1978; Rifkin et la., 2000). The second wave of migrating neural crest cells generates the mediodorsal neurons and the external layer of glial cells in the DRG (Wegner and Stolt, 2005; Wakamutsu et al., 2000; Marmigiere and Ernfors, 2006; Bononi and Bradley, 2006). During this phase nociceptive and thermoreceptive neurons, which express TrkA, are produced (Carr et al., 1978; Rifkin et la., 2000). TrkA and TrkC are receptors for the neurotrophins NGF and NT3, which are important for the survival and axon growth (reviewed in Klein, 1994; Dechant et al., 1994; Barbacid, 1994). In the third phase, cells from the boundary cap migrate through the dorsal roots into the DRG. The boundary cap is a transient cell structure that lies in the meninges of the spinal cord, is derived from the neural crest and emerges in mice at E10.5. Cells of the boundary cap proliferate during embryogenesis and disappear postnatally (Golding and Cohen, 1997; Topilko et al., 1994; Le Douarin et al., 1992; Altman and Bayer, 1984; Hjerling-Leffler et al., 2005). Approximately 5% of the neurons and glial cells of the DRG are derived from the boundary cap (Hjerling-Leffler et al., 2005).

1.2.3 Cranial neural crest derivatives

It has been believed for many years that the facial skeleton is derived from the mesoderm as the skeleton of the whole body. That point of view changed at the end of the 19th century, when it was proposed that the skull is derived from the cranial neural crest rather than from the mesoderm (Platt, 1893). Almost 100 years later it was revealed that not only most parts of the skull are neural crest-derived. It was shown that many other cranial tissues at the facial region are neural crest progeny. Neural crest-derived cranial mesenchymal tissues include the smooth muscles, adipose tissue of skin over the calvarium, skin in the face as well as the ventral part of the neck and many connective components in cephal organs (reviewed in Le Douarin et al., 2004). Neural crest-derived cells were found in each layer of the meninges as well as in the dermis in the forebrain region (Figure 2). In the leptomeninx, a layer between arachnoid and pia mater (Figure 2), neural crest-derived pericytes were detected (Etchevers et al., 1999; Etchevers et al., 2001). Cranial neural crest-derived pericytes were also found at blood vessels in other cranial regions as in the face and ventral neck regions (reviewed in Le Douarin at al., 2004). Pericytes lie adjacent to endothelial cells, the inner layer of the blood vessels, and contribute to the capillary vasoconstriction, and

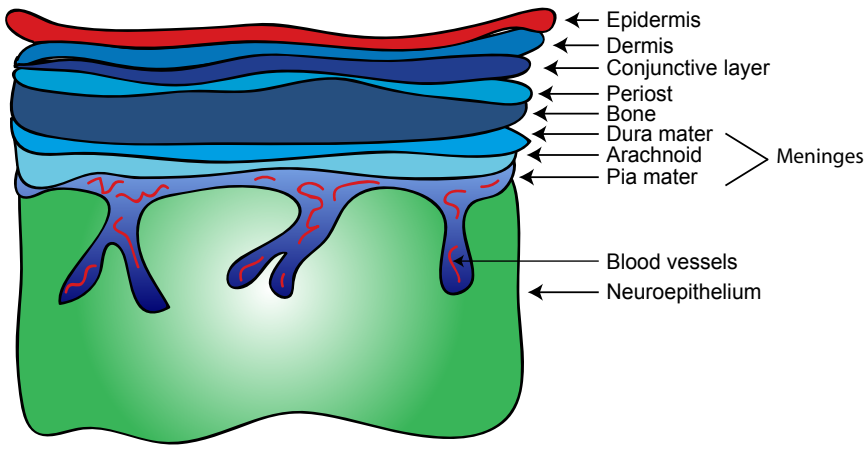

Figure 2: The anatomy of the layers from embryonic anterior head. All blue marked layers contain neural crest-derived cells as in the dermis, conjunctive layer, periost, bone and meninges (dura mater, arachnoid and pia mater). Modified after Etchevers et al., 1999.

to the secretion of specialized extracellular matrices for the microvessels (reviewed in Bergers and Song, 2005). Pericytes in the head are also necessary for the blood-brain barrier (reviewed in Armulik et al., 2011). Endothelial cells of the blood vessels in the head, in contrast to pericytes, are derived from the mesoderm (reviewed in Le Douarin, 2004). Interestingly, trunk neural crest cells do not generate mesenchymal derivatives during normal development with the exception of lower vertebrates, where the dorsal fin is neural crest-derived (Kague et al., 2012).

The cranial neural crest cells also give rise to neurons and glial cells (satellite cells) of the cranial sensory ganglia, parasympathetic ganglia and Schwann cells that myelinate peripheral axons (Le Douarin and Kalcheim, 1999).

1.2.4 Glial cells in the PNS and the development of Schwann cells

During development, the neural crest gives rise to myelinating- and non-myelinating Schwann cells as well as to satellite glial cells (Georgiou et al., 1994; Gershon et al., 1993; Anderson, 1993; Bronner-Fraser, 1993; Le Douarin et al., 1991). Myelinating Schwann cells surround large axons and non-myelinating Schwann cells enclose smaller axons in peripheral nerves. The satellite glial cells are usually found in the ganglia but not in peripheral nerves. They are associated with the cell body of neurons. In the mouse, Schwann cell precursor cells emerge between E12 and E13 (Jessen and Mirsky, 1997) and express the transcription factor Sox10. Sox10 is a transcription factor that binds as all Sox family transcription factors to a specific DNA sequence, the so called *Sry*-Box. It is expressed in migrating neural crest cells, is down regulated in developing neurons but maintained in Schwann cells, plays a role in early stages of the development of Schwann cells and later during the differentiation into myelinating Schwann cells (Britsch et al., 2001; Schreiner et al., 2007). Schwann cell precursor cells express low levels of P0, which is a marker for glial cells in the PNS (Lee et al., 1997). For the survival of Schwann cells, factors secreted from neurons are necessary. Neuregulin, a factor secreted from adjacent neurons, binds to ErbB3/ErbB2 receptors

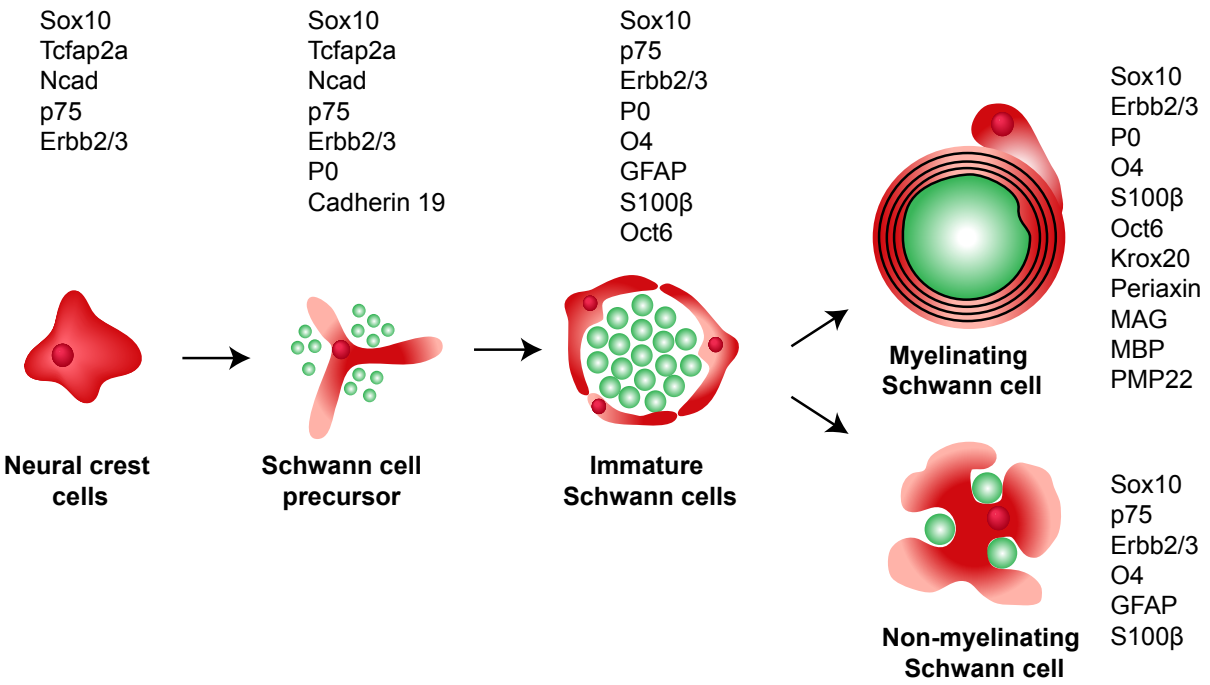

Figure 3: The development of Schwann cells. The neural crest gives rise to many cell types including the Schwann cells of the PNS. Neural crest cells are characterized by their expression of Sox10, Tcfap2a, Ncad, p75 and Erbb2/3. Schwann cell precursor cells additionally express P0 and Cadherin 19. Immature Schwann cells downregulate Tcfap2a and Cadherin 19 as well as start to express O4, GFAP, S100β and Oct6. Immature Schwann cells differentiate either into myelinating or non-myelinating Schwann cells. Myelinating Schwann cells lose p75 as well as GFAP expression and Krox20, Periaxin, MAG, MBP and PMP22 expression is induced. Non-myelinating Schwann cells maintain GFAP and p75 expression but are devoid of myelinating genes as Krox20, Periaxin, MAG, MBP and PMP22. Modified after Jessen and Mirsky, 2005.

and is important for the survival and development of Schwann cell precursor cells (Wolpowitz et al., 2000; Morrissey et al., 1995). Schwann cell precursor cells develop into immature Schwann cells between E13 and E15 in the mouse. Postnatally, immature Schwann cells start to differentiate into myelinating Schwann cells. Tcfap2a and Cadherin 19 are down regulated during the differentiation of Schwann cell precursor cells into mature Schwann cells (reviewed in Jessen and Mirsky, 2005). Immature Schwann cells express O4, GFAP and S100β, which are only weakly or not expressed in Schwann cell precursor cells (reviewed in Jessen and Mirsky, 2005; Woodhoo and Sommer, 2005). Significant expression of GFAP is not detectable until the time of birth (reviewed in Jessen and Mirsky, 2005). Many transcription regulators,

like Krox20, Sox10 and Oct6, are involved during the differentiation of immature Schwann cells to myelinating Schwann cells (Reiprich et al., 2010). Sox10 and Oct6 act together to induce Krox20 expression in vitro (Ghislain and Charnay, 2006). Sox10 and Krox20 then induce the expression of myelin-specific genes like Periaxin, MAG (myelin associated glycoprotein), P0 (protein zero), MBP (myelin basic protein) and PMP22 (proteolipid protein 22). N-CAM, the neurotrophin receptor p75 and GFAP (glial fibrillary acidic protein), which are expressed in immature and non-myelinating Schwann cells, are down regulated during the transition into myelinating Schwann cells (Figure 3) (reviewed in Jessen and Mirsky, 2005). Myelinating Schwann cells are able to dedifferentiate. Adult nerve injuries induce the dedifferentiation of myelinating Schwann cells into immature Schwann cells, which then secrete factors that promote axon regeneration. It is thought that Notch1 plays a major role during this process, as it blocks myelination during the development and is repressed by Krox20 during differentiation (Woodhoo et al., 2007).

1.2.5 The development of the central nervous system (CNS)

The neural tube forms the central nervous system. During neurulation the cranial and caudal parts of the CNS are already committed to their brain and spinal cord fates, respectively. The spinal cord is divided into repeating segments, each with sensory and motor innervation. Before neural tube closure, the three primary vesicles at the cranial region, prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain) are formed, which give rise to all structures of the brain (Figure 4A and B) (reviewed in Nowakowsky and Hayes, 1999).

1.2.6 The development of the neural tube

The neural tube has characteristic dorsoventral areals, induced by morphogen gradients. The development of the ventral part of the neural tube is patterned by a

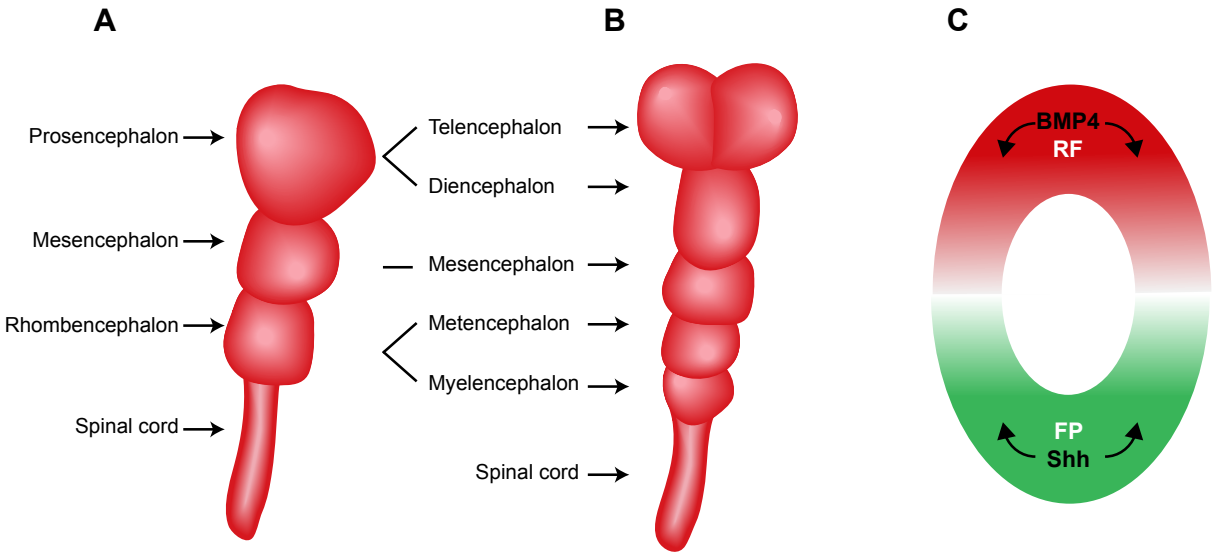

Figure 4: The development of the central nervous system (CNS). (A) The cranial part of the neural tube forms three vesicles: Prosencephalon, mesencephalon and rhombencephalon. (B) Prosencephalon develops to telencephalon and diencephalon, and rhombencephalon becomes metencephalon and myelencephalon. (C) Dorsoventral patterning of the spinal cord by Shh and BMP morphogen gradients results in the differentiation of different types of interneurons at dorsal and ventral regions and motoneurons at the ventral region of the neural tube. Floor plate (FP) and roof plate (RP). Modified after Nowakowsky and Hayes, 1999.

gradient of sonic hedgehog (Shh), secreted from the floor plate and notochord. The floor plate is only found in vertebrates. The ventral interneurons, motoneurons and cells of the floor plate are specified by increasing levels of the Shh morphogen (Figure 4C). At the dorsal region, the BMPs play an essential role for spinal cord pattering. BMPs are initially secreted by the non-neural ectoderm during neural plate generation and induce the generation of the dorsal roof plate. The roof plate also secretes BMPs which leads to the emergence of a BMP morphogen gradient and to the specification of different dorsal interneurons (Figure 4C) (Squire, 2008).

1.2.7 The development of CNS cell types during embryogenesis

The CNS of vertebrates is built of three major kinds of cell types: Neurons, astrocytes and oligodendrocytes. Those three cell types are derived from the neuroepithelium. Neurons, astrocytes and oligodendrocytes are sequentially generated in different parts

of the brain (Altman and Bayer, 1984). In the following, the generation of motoneurons, astrocytes and oligodendrocytes in the spinal cord is described which is of relevance in the context of the present study.

1.2.8 Motoneurons

Motoneurons are responsible for muscle contraction. Degenerative diseases of motoneurons like Amyotrophic lateral sclerosis (ALS) result in muscle atrophy and complete paralysis (Gurney et al., 1994).

The molecular control of motoneuron and interneuron differentiation has been analyzed in great detail, revealing specific cascades by transcription factors that act at different dorso-ventral domains.The ventral spinal cord contains four interneuron domains (p0, p1, p2 and p3) as well as one motoneuron domain (pMN) (Figure 5), in which different types of interneurons and motoneurons are generated. A set of homoeodomain proteins, whose expression is dependent on distinct levels of Shh, determine these distinguishable domains in the ventral spinal cord. The motoneurons are derived from progenitors which are only located at the pMN domain. The pMN domain is defined by the expression of Olig2, Pax6 and Nkx6.1 as well as other genes in adjacent domains like Nkx2.2 and Irx3, which determine the boundary of the pMN domain (Figure 5) (Fu et al., 2002; Zhou et al., 2000; Briscoe et al., 2000, Lu et al., 2000). Olig2 is essential for motoneuron development. It is induced by Nkx6.1 and Nkx6.2 and is sufficient to promote Ngn2 expression, cell cycle exit as well as neuronal marker expression (Bennett et al., 2001; Lui et al., 2003; Novitch et la., 2001; Sun et al., 1998). In vitro it has been shown that Olig2 is induced by FGF2 (reviewed in Rowitch, 2004). The expansion of Olig2 expression outside of the pMN domain is inhibited by the expression of Irx3 (expressed dorsally at the p0, p1 and p2 interneuron domain) and Nkx2.2 (expressed ventrally at the p3 interneuron domain) (Figure 5) (Sun et al., 2003; Lu et al., 2002).

Along the rostral/caudal axis specialized motoneuron subclasses have distinct positions in repeating segments to innervate different peripheral muscles and targets

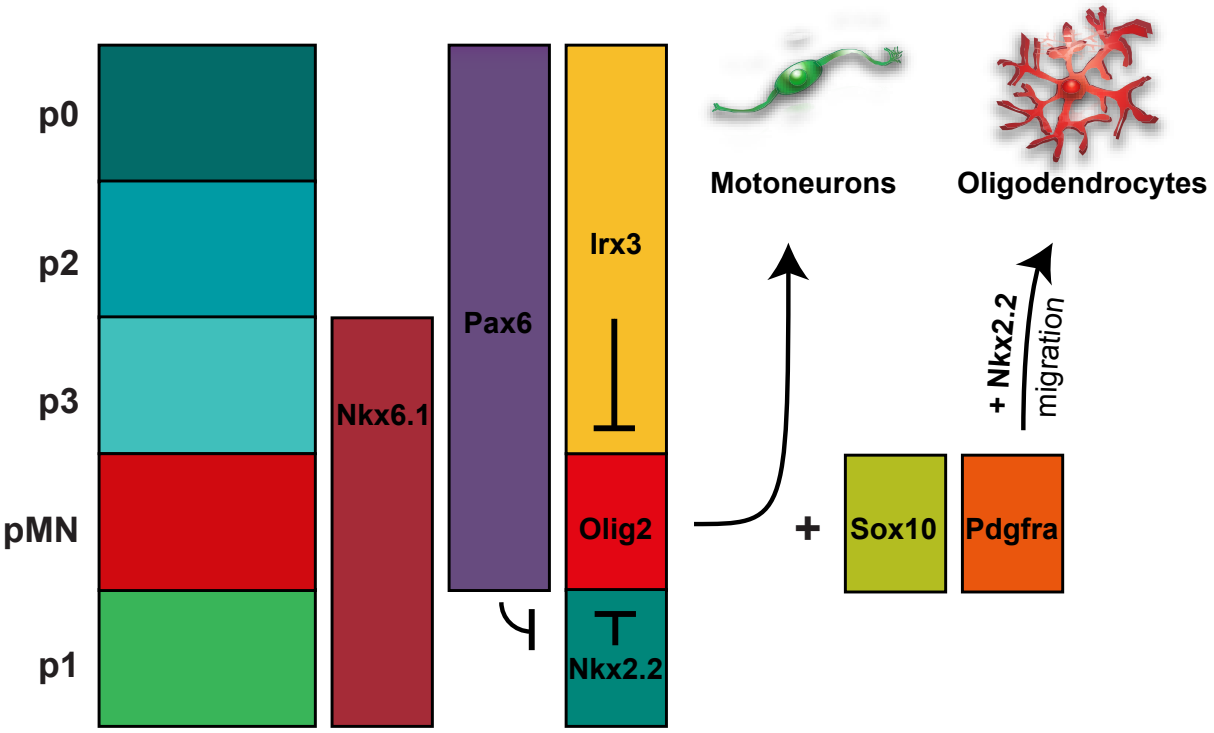

Figure 5: Early spinal cord patterning and development of motoneurons and oligodendrocytes. The motoneuron (pMN) domain sequentially gives rise to motoneurons and to the cells of the oligodendrocyte lineage after motoneuron production. The pMN domain is characterized by the expression of Olig2 and its expansion is inhibited by Irx3 and Nkx2.2. Olig2 is induced through Nkx6.1 and Nkx6.2 and is essential for the induction of neuronal genes in motoneuron development. Dorsal expansion of Nkx2.2 is inhibited by Pax6. In mouse, oligodendrocyte development is induced by Olig2, Sox10 and Pdgfra. After migration starts, OPCs start to express Nkx2.2. Motoneuron domain (pMN); Interneuron domains of the ventral spinal cord (p0, p1, p2, p3).

(reviewed in Lee and Pfaff, 2001). Motoneuron subclasses at the rostral/caudal region are specialized through distinct retinoic acid levels and the Hox gene code (reviewed in Lee and Pfaff, 2001; Dasen et al., 2003).

1.2.9 Astrocytes

Astrocytes are responsible for the composition of the extracellular environment in the CNS. They have many receptors for neurotransmitters and control the ion concentration in extracellular spaces by gap junctions and ion channels (Kettenmann et al.,

1984; Brightman and Reese, 1969). It is thought that astrocytes play a major role in the establishment of functional synapses (Ullian et al., 2001). Astrocytes, like late immature- and non-myelinating Schwann cells, express GFAP. GFAP is a marker for astrocytes in the CNS (Bignami et al., 1972; Walz and Lang, 1998). In the spinal cord astrocytes are generated at all dorsal and ventral regions, except for the motoneuron region, where only motoneurons and oligodendrocytes are generated (Pringle et al., 1998).

1.2.10 Oligodendrocytes

The majority of oligodendrocytes populate the white matter tract and generate myelin for the isolation of axons to allow fast impulse propagation (Bunge, 1968). Dysfunction of myelin through injury, pathological degeneration (Waxman, 1991) or genetical ablation (Nave, 1995) leads to serious defects in the CNS. For example, focal myelin degeneration in the disease of Multiple sclerosis results in the progressive loss of the neuronal functions in the CNS (Waxman, 1991).

In the following section the development of oligodendrocytes in the spinal cord will be delineated, because it belongs to the best understood models. Oligodendrocyte precursor cells (OPCs) develop within the pMN domain at a selective time window, starting after motoneuron development (Lu et al., 2000; Richardson et al., 1997; Spassky et al., 2000). In the mouse, oligodendrocyte precursor cells are born at E12.5 and start to express Pdgfra and Sox10. Early oligodendrocyte progenitor cells start to migrate to the dorsal and ventral parts of the spinal cord at E13.5 and start to express Nkx2.2 (Fu et al., 2002; Zhou et al., 2001). At about E13.5 oligodendrocyte precursor cells are also generated at the dorsal spinal cord and are not regulated by Shh signaling (Vallstedt et al., 2005, Cai et al., 2005). Late OPCs (Figure 6) start to express the POA antigen, which is recognized by O4 antibody. However, O4-positive OPCs are still proliferative until the onset of the final differentiation (Bansal and Pfeifer, 1992; Warrington et al., 1993; Ono et al., 2001). During maturation, oligodendrocytes extend multiple

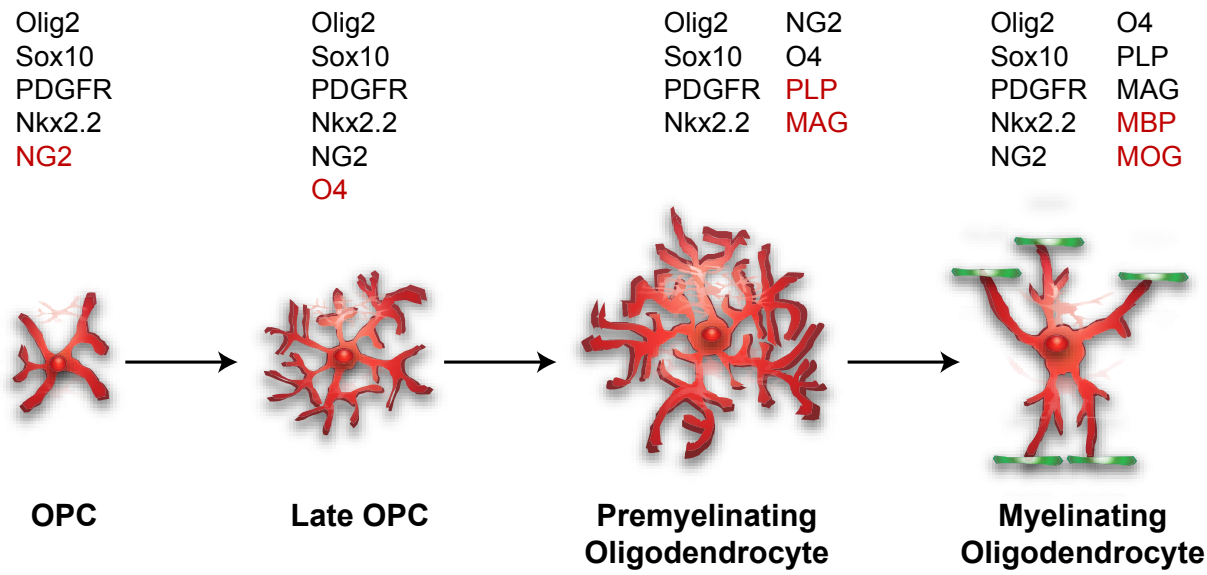

Figure 6: The differentiation of oligodendrocytes. Oligodendrocyte precursor cells (OPCs) are characterized by their expression of Olig2, Sox10, Pdgfra, Nkx2.2 and NG2. O4 expression is induced in late OPCs. Premyelinating oligodendrocytes additionally express PLP and MAG. MBP and MOG are expressed in Myelinating oligodendrocytes.

processes for later myelination. Contrary to Schwann cells, where each Schwann cell myelinates a single axon, oligodendrocytes are able to myelinate many axons with their processes. During this differentiation, oligodendrocytes mature into premyelinating oligodendrocytes that express MAG, CNP and MBP (Figure 6). Myelinating oligodendrocytes start to express PLP and MAG (Figure 6) (reviewed in Nicolay et al., 2007; reviewed in Miller, 2002; reviewed in Zhang, 2001; reviewed in Woodruff, 2001). Like in Schwann cells, Sox10 plays a major role in the induction of genes that are important for myelination (Stolt et al., 2002; Liu et al., 2007).

1.3 Stem cells in the developing and adult tissue

A stem cell is a cell which is characterized by the ability to divide throughout the lifetime of an organism and to produce both stem cells (self-renewal) and differentiated daughter cells. Thus, stem cells are identified by their potential to self-renew, based on the production of at least one equivalent daughter cell. Furthermore, they have a more

or less broad developmental potential, which means that they are able to differentiate into different cell types. Thus, stem cells can be subdivided into different categories depending on their developmental potential. A totipotent stem cell, the zygote, is able to form a whole organism. Pluripotent stem cells have the potential to differentiate into all cell lines of an organism. Therefore, they differentiate into cells of all three germ layers. They are described as embryonic stem cells (ESCs) and are not able to form an entire organism, in contrast to totipotent stem cells. Multipotent stem cells have a restricted developmental potential and are able to generate multiple cell lines that constitute an entire tissue/organ. Oligopotent stem cells form at least two cell lines within a specific tissue. Unipotent stem cells form only one cell line or cell type within a specific tissue (Smith, 2006).

ESCs are defined as pluripotent stem cells and can be isolated from the morula or from the inner part of the blastocyst. Recent findings describe the production of induced pluripotent stem cells (iPSCs) derived from fibroblasts (Takahashi et al., 2006; Takahashi et al., 2007; Park et al., 2007; Yu et al., 2007) which will be described in more detail below. An alternative to ESCs and iPSCs for cell replacement therapies are tissue-specific stem cells. Tissue-specific stem cells populate embryonic-, postnatal- and adult tissues and function in maintenance and repair of relevant tissues (Smith et al., 2006).

1.3.1 Induced pluripotent stem cells (iPSCs)

The work with human ESCs is of high interest because of their potency to generate cells of all three germ layers and their potential use to treat degenerative diseases by transplantation. However, the use of human ESCs is an ethical issue which is discussed controversely in different countries. In Germany, the isolation and generation of human ESCs is forbidden by law to secure the human dignity and life protection. The begin of human life starts after egg fertilization and the generated zygote is already under the protection of human dignity in Germany. The use of human ESCs for cell replacement

therapies is also complicated because of tissue rejection after the transplantation of heterologous human ESCs into patients (Swijnenburg et al., 2005; Nussbaum et al., 2007; Bonnevie et al, 2007). The generation of iPSCs avoids these problems because it allows the generation of autologous stem cells from adult tissue. In 2006 the research group of Yamanaka showed for the first time the successful reprogramming of mouse embryonic and adult fibroblasts into induced pluripotent stem cells (iPSCs) by retroviral introduction of a group of defined factors i.e. Oct3/4, Sox2, c-Myc and Klf4, which were known for their role in the maintenance of pluripotency in ESCs (Takahashi et al., 2006). One year later the same group demonstrated the generation of iPSCs derived from adult human dermal fibroblasts by the same factors (Takahashi et al., 2007). Simultaneously, several other groups confirmed these findings (Park et al., 2007; Yu et al., 2007). The advantage of iPSCs is that they can be produced from patient tissue and that transplanted iPSCs are not rejected. However, like ESCs also iPSCs generate teratomas after transplantation into syngeneic or immunodeficient mice and teratoma will also be formed after transplantation of human iPSCs into patients (reviewed in Ben-David and Benvenisty, 2011). Another disadvantage represents the retrovirally introduced transgenes in iPSCs. At the present stage iPSCs cannot be used for stem cell based therapy because of safety concerns (Aoi et al., 2008). Transient transgene introduction via expression plasmids, episomal vectors or *Piggy Bac* transposon are expected to avoid permanent genetic alteration during iPSC induction. In addition, cultured iPSCs tend to genomic alteration by acquiring chromosomal trisomy which leads to enhanced proliferation and is probably linked to the culture conditions used (reviewed in Ben-David and Benvenisty, 2011). An alternative for the generation of specific cell types from iPSCs is the direct reprogramming of fibroblasts to specific cell types without pluripotent state. Recent investigations describe the generation of induced neural stem cells (iNSCs) from fibroblasts by defined factors (Han et al., 2012). iNSCs show the potential to self-renew, display nearly identical features to control NSCs and are able to differentiate into neurons, astrocytes and cells of the oligodendrocyte lineage in vitro and in vivo (Han et al., 2012). Other groups demonstrated the generation

of oligodendrocyte-like cells from fibroblasts by ectopic expression of the transcription factors, Sox10, Olig2 and Zfp536 or Nkx6.1. Induced oligodendrocyte-like cells show the potential to mature into myelinating oligodendrocytes after the transplantation into hypomyelinated and *shiverer* mice (Yang et al., 2013; Najm et al., 2013). It remains unclear, however, whether tumors develop from the implanted cells.

1.3.2 Tissue-specific stem cells

Tissue-specific stem cells populate fetal and adult tissue and function as a cell reservoir. During embryonic development, the cells of the germ layers undergo multiple steps of proliferation and the progeny differentiate into multipotent, tripotent, bipotent and unipotent precursor cells in the organism. The majority of the cells develops into differentiated progeny, whereas a small fraction of the cells remains as stem cells in the specific environment (stem cell niche) and has a restricted developmental potential (reviewed in Young and Black, 2004). For example hematopoietic stem cells populate the bone marrow and are able to differentiate into all mature blood cells (reviewed in Wilson and Trumpp, 2006). Myofibroblasts contribute to the maintenance and repair of muscle tissue (Mauro, 1961). Additionally, the small intestine, which includes the duodenum, jejunum and ileum, harbours stem cells that show the ability to renew the epithelium within a time period of 5 days (reviewed in Barker et al., 2008). Tissue-specific stem cells were identified in many postnatal and adult tissues (reviewed in Young and Black, 2004) and some of them will be described below.

1.3.3 Mesenchymal stem cells

At least two distinct stem cell populations, the hematopoietic stem cells and the mesenchymal stem cells (MSCs), also called multipotent marrow stromal cells or mesenchymal stromal cells, reside in the bone marrow stroma. Besides the bone marrow stroma, which is the first tissue analyzed for MSC isolation, cells with MSC

characteristics can be isolated from other mesodermal-derived tissues such as the peripheral blood, fetal liver and lung, adipose tissue, skeletal muscle, amniotic fluid, synovium and the circulary system (reviewed in Wagey, 2011). MSCs are defined by their ability to self-renew, and their multipotent developmental potential, which means the ability to differentiate in several mesenchymal cell types (reviewed in Caplan, 1991). There is a considerable interest to use MSCs in regenerative medicine because of their ability to repair damaged muscle including the ischemic heart muscle (reviewed in Grove et al., 2004). To standardize such approaches the International Society for Cellular Therapy defined minimal criteria of human MSCs for cellular therapy. MSCs should have the following characteristics: *i)* They must be adherend on plastic. *ii)* They must express certain surface antigens, such as CD105, CD73 and CD90, and must be devoid of CD45 (pan-leukocyte marker), CD35 (marker for primitive hematopoietic progenitor cells), CD14 and CD11b (markers for monocytes and macrophages) as well as CD79α and CD19 (markers for B cells). Finally, they must be able to differentiate into osteoblasts, adipocytes and chondroblasts in vitro. These criteria only apply for human MSCs and are not well characterized for murine MSCs (Dominici et al., 2006). Notably, many if not all of the markers are also expressed by cultured pericytes (Crisan et al., 2008). Furthermore, long term cultured pericytes are able to differentiate into chondrocytes, adipocytes and osteocytes in vitro, having the same developmental potential like MSCs. It turned out that endogenous pericytes within the tissue express all the above mentioned MSC markers, which raises the question if MSCs originate from pericytes (Crisan et al., 2008). Indeed, it was even suggested that all MSCs are pericytes (reviewed in Caplan, 2008). This is supported by the finding that MSCs derived from endometrium express pericyte markers such as CD146 and PDGF-Rβ (Schwab and Gargett, 2007). An alternative possibility would be that pericytes represent a subpopulation of MSCs (Blocki et al., 2013). Although the origin of MSCs, even if heterogeneous, is presently not clear, a close similarity of MSC and Pericyte identity is evident.

1.3.4 Stem cells in the central nervous system (CNS)

There are two very well described adult stem cell niches in the CNS, the subgranular zone of the dentate gyrus and the subventricular zone (SVZ). CNS stem cells in the SVZ display characteristics of radial glial cells and astrocytes, reflected by their expression of GFAP and Nestin (Merkle et al., 2004). They generate neuroblasts in the SVZ that migrate through the rostral stream into the olfactory bulb where they differentiate into interneurons (Figure 7). These stem cells contribute to the maintenance of the olfactory system in vivo (reviewed in Louis, 2013). Stem cells, which are described as radial glia-like cells, populate the subgranular zone of the dentate gyrus and express, like SVZ stem cells, GFAP and Nestin (Seri et al., 2001). They generate mature granule cells after many steps of asymetric and symetric dividing (Figure 7). The generation of mature granule cells takes about 4 to 6 weeks (Zhao et al., 2007) and it is thought that newborn mature granule cells are required for processing new memory (Deisseroth et

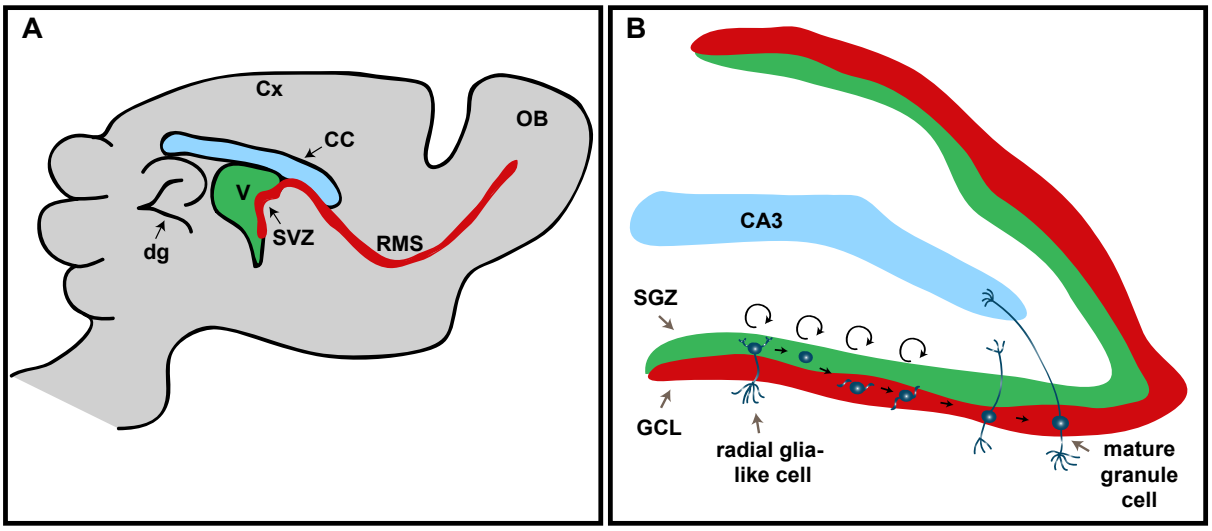

Figure 7: Stem cell niches in the CNS. The two major stem cell niches in the CNS are the subventricular zone (SVZ) (A) and the subgranular zone (SGZ) of the dentate gyrus (dg)(B). (A) Stem cells of the SVZ migrate through the rostral migratory stream (RMS) into the olfactory bulb (OB) where they generate interneurons. (B) Radial glia-like cells in the subgranular zone of the dentate gyrus generate mature granule cells after many steps of asymetric and symetric dividing. Dentate gyrus (dg); Ventricle (V); Corpus callosum (CC); Cortex (Cx); Granule cell layer (GCL); CA3 region of the dentate gyrus (CA3). (A) Modified after Saha et al., 2012; (B) modified after Hanson et al., 2011.

al., 2004; Wiskott et al., 2006). In vitro, stem cells isolated from the adult CNS generate neurospheres (NS), and show stem cell properties. They have an at least oligopotent developmental potential, demonstrated by the differentiation into neurons, astrocytes and oligodendrocytes, and have the ability to self-renew (Reynolds and Weiss, 1992; Richards et al., 1992).

Different research groups found stem cell niches in other postnatal and adult regions of the CNS. Cells which are isolated from postnatal and adult cerebellum display stem cell characteristics by using the neurosphere assay (Lee et al., 2005; Klein et al., 2005). Furthermore, stem cells were isolated from the adult spinal cord, which have the ability, as SVZ-derived NS, to self-renew and differentiate into neurons, oligodendrocytes and astrocytes in vitro (Weiss et al., 1996). The isolation of CNS stem cells and the generation of CNS cell types is of considerable interest for the potential use in regenerative medicine to treat diseases like Multiple sclerosis, Parkinson, Alzheimer and others.

1.3.5 Neural crest stem cells (NCSCs)

The first in vivo evidence for a multipotent state of neural crest stem cells (NCSCs) was obtained from lineage tracing experiments, where it was shown that the progeny of individual dye-labeled neural crest cells migrate and differentiate into cell types of the DRG, sympathetic ganglia, Schwann cells of ventral roots, as well as into neuroepithelial cells and neurons of the neural tube in vivo (Bronner-Fraser and Fraser, 1989). These results were supported by the findings from Frank and Sanes in 1991, who showed that the progeny of single *LacZ*-carrying retrovirus-labeled NCSCs are able to differentiate into both, neurons and glial cells of the DRG (Frank and Sanes, 1991). Earlier findings that the facial skull and other facial mesenchymal tissues in the cranial region are neural crest-derived were supported by using several transgenic mouse lines, expressing *Cre* under the control of neural crest gene promotors such, as *Wnt1*, *P0* and *Sox10*, and so activating *GFP* or *LacZ* expression (reviewed in Dupin

and Douarin, 2014). Additionally, a multipotent state of NCSCs was also demonstrated in vitro, using clonal cultures that generate colonies from cephalic NCSCs that contain several cell types like neurons, glial-, pigment cells and cartilage and show the potency to self-renew (Baroffio et al., 1988; Baroffio et al., 1991).These results underline that NCSCs have a high developmental plasticity, reflected by their differentiation into cells of the peripheral nervous system and mesenchymal lineage. An important breathrough in the analyses of neural stem cells came with the studies of Reynolds and Weiss in 1992. They showed that neural stem cells generate neurospheres (NS) in vitro under specific medium conditions containing growth factors such as EGF and FGF (Reynolds and Weiss,1992; Reynolds and Rietze, 2005). One single NS is generated by a single stem cell, which produces daughter cells throughout continuous dividing that leads to the emergence of a spherical structure. Since cultured NS are able to fuse with each other, and two fused NS with a unipotent developmental potential would mimic a broader developmental potential, clonal NS assays are used to avoid this problem (Sinseg et al., 2006; Jessberger et al., 2007; Mori et al., 2007; Coles-Takabe et al., 2008). Clonal NS can be generated by very low density cultures to avoid NS fusion or by seeding one single cell in one well. The multipotent state of NCSCs was only confirmed in a few clonal cultures, arguing that the cells need different environmental cues for differentiation (Baroffio et al., 1988; Baroffio et al., 1991; reviewed in Sommer, 2001). It was shown that certain factors promote cell type specific differentiation of NCSCs. For example TGF-β promotes smooth muscle differentiation, BMP2 autonomic neuron differentiation, NRG1 glial differentiation and Wnt1 sensory neuron differentiation (reviewed in Shakhova and Sommer, 2010). Furthermore, multipotent and self-renewing NCSCs can be isolated via the low affinity NGF receptor p75 (Stemple and Anderson, 1992) and Sox10 is required for the survival of NCSCs (Paratore et al., 2001). Interestingly, NCSCs are found in many postmigratory tissues (Dupin et al., 2007) and some of them will be described below.

1.3.6 Postmigratory NCSCs

NCSCs reside in many embryonic, postnatal and adult postmigratory neural crest-derived tissues. In 1992, the group of Sieber-Blum successfully isolated multipotent cells from quail embryonic DRG and the sympathetic ganglion (Duff et al., 1992). Since the DRG and the sympathetic ganglion are composed of many different cell types, the identity of those multipotent stem cells was unknown. Convincing evidence for the existence of postmigratory NCSCs was obtained by the Anderson lab. The group isolated p75-positive, P0-negative, multipotent and self-renewing NCSCs from E14.5 rat sciatic nerve that generate neurons and glia when transplanted into chicken embryos (Morrison at al., 1999). These results were supported by the finding that p75-positive multipotent NCSCs can also be isolated from embryonic and adult rat gut (Kruger et al., 2002). Several other groups confirmed the existence of multipotent, self-renewing postmigratory trunk NCSCs (Hjerling-Leffler et al., 2005; Li et al., 2007; Nagoshi et al., 2008).

Cranial NCSCs often have a broader developmental potential in comparison to postmigratory trunk NCSCs, because of their potency to generate mesenchymal derivatives like osteocytes, chondrocytes and adipocytes. Cranial NCSCs were found in the olfactory mucosa, respiratory mucosa, oral mucosa, eye, periodontium and the palate (reviewed in Kaltschmidt et al., 2011).

Since the trunk neural crest has a more restricted developmental potential in comparison to the cranial neural crest, the detection of in vitro generated mesenchymal derivatives from trunk-derived NCSCs, under defined medium conditions, was surprising (Calloni et al., 2007; Calloni et al., 2009; Ido et al., 2006). John et al., (2011) demonstrated that the generation of mesenchymal derivatives from the trunk neural crest is TGF-β dependent (John et al., 2011).

1.3.7 Easily accessible tissue sources for NCSC isolation

It is of considerable interest to isolate NCSCs from easily accessible tissues with respect to their potential use in regenerative medicine. An easily accessible tissue for NCSC isolation represents the facial skin. Using a *Wnt1-Cre/R26R* mouse to trace the localization of NCSCs, galactosidase-positive signals were found in whisker papilla cells, mainly at the bulge region (Sieber-Blum et al., 2004). Those bulge-derived cells which also express Nestin and Sox10, have the ability to self-renew as well as to differentiate into neurons, Schwann cells and into chondrocytes (Sieber-Blum et al., 2004).

An additional easily accessible tissue source for NCSC isolation is the adult rat palatal rugae. Nestin- and p75-coexpressing NCSCs, which lie adjacent to Meissner corpuscles and Merkel cell-neurite complexes, were detected (Widera et al., 2009). These NCSCs are also colocalized within neurofilament-M-positive axons. So called palatal neural crest-related stem cells (pNCSCs) could be cultured by using the neurosphere (NS) assay. Newly propagated pNCSCs revealed the expression of neural crest markers, such as Sox9, Twist, Slug and p75. Those cells also express the stem cell markers CD133 and Nestin as well as pluripotency genes, such as Sox2, Oct4, Klf4 and c-Myc. Furthermore, pNCSCs were able to differentiate into neurons, GFAP-expressing glial cells and smooth muscle cells in vitro. The potency to generate mesenchymal derivatives from pNCSCs was not analyzed (Widera et al., 2009; Widera et al., 2011)

1.3.8 NCSCs have the potential to generate CNS derivatives

Postmigratory NCSCs display a high developmental plasticity shown by their differentiation into cells of the peripheral nervous system as well as into cells of the mesenchymal lineage. First evidence for the plasticity of DRG progenitors to generate CNS progeny came from Svenningsen and colleagues (2004). They revealed that satellite glial cells of the DRG show the potential to generate OPCs that express Ng2

as well as Pdgfra and generate GFAP-expressing astrocyte-like cells (Svenningsen et al., 2004). In addition, Dromard et al., (2007) detected Olig2-expressing cells in NS cells derived from E13.5 DRG that were cultured in the presence of high amounts of FGF and EGF.

Furthermore, parallel studies by Baron-Van-Evercooren and Rohrer demonstrated that NCSCs, derived from postmigratory tissues, show the ability to differentiate into CNS cell types in vitro and in vivo (Zujovic et al., 2009; Binder et al., 2011; Zujovic et al., 2011) The group of Baron-Van-Evercooren showed that E10.5 boundary cap cells transplanted into the spinal cord display CNS characteristics. Transplanted boundary cap cells express CC1, Olig2 and MOG, which are usually expressed in cells of the oligodendrocyte lineage (Zujovic et al., 2009). The group of Rohrer found out that NCSCs derived from E12.5 mouse DRG display CNS characteristics in culture and are able to differentiate into CNS cell types in vitro and in vivo. After transplantation into brains of postnatal *shiverer* mice, the injected cells predominantly differentiated into cells of the oligodendrocyte lineage, including myelinating oligodendrocytes (Binder et al., 2011). Similar findings were obtained from the group of Baron-Van-Evercooren. They showed that boundary cap derived NCSCs generate CNS oligodendrocytes in vitro and in vivo (Zujovic et al., 2011). Even adult human skin-derived NCSCs differentiate into dopaminergic neurons by different combinations of supplemented factors (Narytnyk et al., 2014). Taken together, these findings demonstrate convincingly that NCSCs acquire CNS fates and thus may represent a potential source for CNS stem cell based therapies.

2. Aim of the project

Postmigratory neural crest-derived stem cells (NCSCs) display a high developmental potential, reflected by their differentiation into cells of the PNS and cells of the mesenchymal lineage. In addition, the generation of CNS cell types from NCSCs were recently described (Zujovic et al., 2009; Binder et al., 2011; Zujovic et al., 2011; Narytnyk et al., 2014).

Sox10-positive NCSCs from E12.5 mouse DRG cultured in the presence of EGF and FGF show the ability to self-renew, generate neurospheres (NS) and have an at least oligopotent developmental potential. Passage 3 NCSC NS express the CNS transcription factor Olig2 and are able to differentiate into CNS cell types, including neurons, oligodendrocytes and astrocytes in vitro (Binder et al., 2011). PNS marker genes, such as Peripherin and p75, are undetectable in passage 3 NS (Binder et al., 2011, Marlen Weber, Diploma thesis, 2010). In vivo, it has been demonstrated that the majority of DRG-derived NCSCs differentiate into cells of the oligodendrocyte lineage upon injection into embryonic, postnatal and adult mouse brains (Binder et al., 2011). These DRG-derived NCSCs acquire a ventral spinal cord identity in NS culture, as it is indicated by the expression of trunk Hox genes, ventral neural tube marker genes and the lack of cortical markers (Binder et al., 2011). It is, however, unclear whether the reprogramming of NCSCs to rNCSCs (reprogrammed neural crest-derived stem cells) results in a partial or complete CNS identity.

The aim of the project is to analyze the reprogramming of embryonic DRG-derived NCSCs towards CNS fates in more detail. This includes the examination of the molecular and cellular mechanism of the reprogramming process in vitro. Gene expression profiles of rNCSCs and spinal cord stem cells (SCSCs) are expected to give detailed information of the gene expression pattern of rNCSCs in comparison to SCSCs.

Furthermore, it is aimed to establish a culture system, that prevents the reprogramming process of DRG-derived NCSCs. The differentiation potential and the cell type

identity of those NCSCs will be analyzed.

The use of rNCSCs for cell therapies requires an accessible source of these cells in the adult organism. As the DRG from adult mice is not an easily reachable tissue, it was of interest to identify an alternative source. An easily attainable tissue source that contains NCSCs is the adult rat palatal rugae (Widera et al., 2009; Widera et al., 2011). It is aimed to investigate whether NCSCs from adult mouse palate acquire a CNS identity in the presence of EGF and FGF like embryonic DRG-derived rNCSCs. Additionally, using a *Sox10-GFP* mouse line, the cell type identity of palate-derived NS-forming cells will be examined.

3. Materials and Methods

3.1 Materials

3.1.1 List of manufacturers

Manufacturer	Chemical/Substance
Applied Biosystems, Carlsbad, California	Random Hexamer Primer
Prepro Tech EC, London, UK	NGF
Boehringer, Mannheim, Germany	DTT
Cell Systems Biotech. Vertrieb GmbH, St. Katharinen, Germany	Collagenase (220 U/mg)
Charles River Deutschland GmbH, Extertel, Germany	C57Bl/6J mice
Difco, Detroit, Michigan, USA	Bacto-Yeast-Extract, Bacto-Trypton, Bacto-Agar
Fisher Scientific, Nidderau, Germany	Omnifix single-use syringe, canulaes
Gibco BRL, Invitrogen, Life Technologies, Eggenstein, Germany	Agarose, L-Glutamine, Fetal Calf Serum (FCS), Penicillin-Streptomycin (5000 U/ml), Trypsin-EDTA, β-Mecaptoethanol, dNTP Set (PCR), Laminin, M-MLV Reverse Transcriptase, M-MLV Reverse Transcriptase Kit, N2-Supplement (100x), B27-Supplement (50x), bFGF, EGF, Trypsin-EDTA, Oligo dT20-Primer, RNase H, RNase out, secondary antibodies, Super Script III Reverse Transcriptase, Super Script III Polymerase Kit, RT Kit, Taq DNA-Polymerase kit, 1 kb plus ladder, ELECTROMAX™ DH5α-E™ Component cells, Trizol, MEM
Merck, Millipore, Darmstadt, Germany	Boric acid, CaCl x $2H_2O$, Glucose, $MgSO_4$ x $7H_2O$, NaCl, $NaHCO_3$, Na_2HPO_4, LiCl, KH_2HPO_4, rabbit anti-Olig2, Paraformaldehyde
MWG Biotech., Ebersberg, Germany	Oligonucleotides (Primer)
Nunc, Wiesbaden, Germany	35 mm cell-culture dishes
Qiagen, Hilden, Germany	Gel extraction kit, Plasmid Purification Midi and Mini kit, Qiashredder-columns, RNeasy Mini Kit, PCR Purification Kit, HotStarTaq Plus Master Mix Kit
R&D Systems, Wiesbaden, Deutschland	BMP4, Neuregulin1, NT3, goat anti-Sox3
Riedel-De-Haen, Seelze, Germany	Ethanol, NaOH, HCl, Trinatriumcitrate
Roche Diagnostics, Mannheim, Germany	Restrictionendonucleases (with matching Buffers), Proteinase K, Ampicillin
Roth, Karlsruhe, Germany	Triton-X-100
Serva, Heidelberg, Germany	Paraformaldehyde, Bromphenolblue
Sigma-Aldrich-Chemicals, Taufkirchen, Germany	DMEM/F12, DNase1, Ethidiumbromid, HEPES, Isopropanol, Poly-DL-Orinithine, Hydrobromid, Bovine-Serum-Albumin (BSA), Yeast RNA, Xylencyanol, Heparin, Forskolin, Ascorbic acid sodium, cAMP, Retinoic acid (RA)
VWR, Darmstadt, Germany	Glass Cover Slips (10 mm Diameter), Pasteurpipettes
Worthington, Freehold, Ohio, USA	Trypsin, Soybean-Trypsin-Inhibitor
Promega, Mannheim, Germany	pGEM®-T Easy vector system, BDNF, GDNF, rabbit anti-p75
Lonza AG, Köln, Germany	Amaxa™ Basic Neuron SCN Nucleofector ™ Kit

J.T.Baker, Deventer, Netherland	Potassium chloride (KCl)
Becton Dickson, Le Pont de Claix, France	Petri-dishes (60x15 mm, 35x10 mm)
BD Falcon, Franklin Lakes, NJ, USA	Cell strainer (40 µm, 100 µm), reaction tubes (50 ml, 15 ml)
Greiner-Bio-One GmbH, Frickenhausen, Germany	Cell culture flasks, 4 well-plates
PAA Laboratories GmbH, Cölbe, Germany	Accutase, Amphotericin B
Abcam, Cambridge, UK	Mouse anti-p75, Mouse anti-Peripherin
Santa Cruz Biotechnology, Inc., Heidelberg, Germany	Goat-anti Sox10
Hiss Diagnostics GmbH, Freiburg, Deutschland	Mouse and rabbit anti-Tuj1
Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA	Mouse anti-Tcfap2a
Cell Signaling Technology, Leiden, Netherland	Rabbit anti-CNPase
Sanofis Aventis, Frankfurt, Germany	DAPI
SARSTEDT, Nümbrecht, Germany	Petri-dishes, 10 mm

Table 1: List of all chemicals and substances and the according manufactures

3.1.2 Media, solutions and supplements

If not stated otherwise, media and solutions were made with highly purified H_2O (MilliQ-filter system by Millipore).

3.1.2.1 Media used for cell culture

All media were sterile filtrated

Medium	Composition
Proliferation medium for rNCSCs, SCSCs, and NCSCs from postnatal (P3) mice	DMEM/F12 1:1 1% N2-Supplement 1% B27-Supplement 0.7% L-Glutamine 1% Penicillin/Streptomycin (P/S) 20 ng/ml bFGF 20 ng/ml EGF 0.5 U/ml Heparin
Proliferation medium for FGF NCSCs and FGF SCSCs	DMEM/F12 1:1 1% N2-Supplement 1% B27-Supplement 0.7% L-Glutamine 1% P/S 20 ng/ml bFGF 0.5 U/ml Heparin
Proliferation medium for EGF SCSCs	DMEM/F12 1:1 1% N2-Supplement 1% B27-Supplement 0.7% L-Glutamine 1% P/S 20 ng/ml EGF 0.5 U/ml Heparin

Proliferation medium for BMP NCSCs	DMEM/F12 1:1 1% N2-Supplement 1% B27-Supplement 0.7% L-Glutamine 1% P/S 20 ng/ml bFGF 20 ng/ml EGF 0.5 U/ml Heparin 10 ng/ml BMP4
Proliferation medium for pNCSCs	DMEM/F12 1:1 3% B27-Supplement 1% L-Glutamine 1% P/S 1% Amphotericin b 40 ng/ml bFGF 20 ng/ml EGF 0.5 U/ml Heparin
Standard differentiation medium	DMEM/F12 1% B27-Supplement 1% L-Glutamine 1% P/S 1.5% FCS 50 nM retinoic acid (RA)
Schwann cell differentiation medium	DMEM/F12 1% B27-Supplement 1% L-Glutamine 1% P/S 1% FCS 4 µM forskolin 50 ng/ml neuregulin 1 (NRG1)
Medium, containing EGF and FGF, for neuronal differentiation of BMP NCSCs	DMEM/F12 1:1 1% N2-Supplement 1% P/S 1% L-Glutamine 10 ng/ml bFGF 10 ng/ml EGF
Neuronal differentiation medium of BMP NCSCs	DMEM/F12 1:1 1% N2-Supplement 1% P/S 1% L-Glutamine 10 ng/ml BDNF 10 ng/ml GDNF 10 ng/ml NT3 10 ng/ml NGF 200 µM ascorbic acid 0.5 mM cAMP
BSA in DMEM/F12	3% (w/v) BSA in DMEM/F12

Table 2: List of all used cell culture media

3.1.2.2 Solutions and Supplements used for cell culture

Solutions	Composition
Trypsin-EDTA	0.05% (w/v) Trypsin, 0.02 g (v/v) EDTA in HBSS
0.15 M Borate buffer	9.28 g Boric acid dissolved in 1 l H_2O; pH adjusted to pH 8.3 with 30% NaOH; autoclaved
Poly-DL-ornithine in Borate Buffer	0.5 mg/ml Poly-DL-ornithine in 0.15 M Borate buffer, steril filtered
Trypsin solution	1890 U/ml Trypsin disssolved in PBS; sterile filtrated; stored at -20°C, before use diluted 1:10 in PBS
Collagenase	250 U/ml Collagenase dissolved in PBS; sterile filtrated
DNase1	2mg/ml DNAse1 in PBS; sterile filtrated; stored at -20, before use diluted 1:100 with medium
Soybean-Trypsin-Inhibitor	5.8 mg/ml Soybean-Trypsin-Inhibitor dissolved in PBS; sterile filtrated; stored at -20°C, before use diluted 1:20 with medium
10x PBS	1.3 mM NaCl, 40 mM Na_2HPO_4, 14 mM KH_2PO_4, 24 mM KCl, pH 7.3
PBS/Glucose	1 mg/ml Glucose in PBS; sterile filtrated
Amphotericin B	250 µg/ml
B27-Supplement	50% (stocksolution)
N2-Supplement	100% (stocksolution)
bFGF (human recombinant)	20 ng/ml, 40 ng/ml (100 ng/µl stocksolution)
EGF (mouse)	20 ng/ml (200 ng/µl stocksolution)
Heparin sodium salt	10.000 U/ml (stocksolution)
L-Glutamine	200 mM (stocksolution)
BMP4 (human, recombinant)	10 ng/ml (40 ng/µl stocksolution)
BDNF (human, recombinant)	10 ng/ml (20 ng/µl stocksolution)
GDNF (human, recombinant)	10 ng/ml (20 ng/µl stocksolution)
NT3 (human, recombinant)	10 ng/ml (20 ng/µl stocksolution)
NGF (human, recombinant)	10 ng/ml (20 ng/µl stocksolution)
Forskolin (human, recombinant)	4 µM (10mM stocksolution)
Neuregulin1 (human, recombinant)	50 ng/ml (100 ng/µl stocksolution)
cAMP	500 µM (50 mg/ml stocksolution)
Ascorbic acid	200 µM

Table 3: List of all cell culture supplements and solutions

3.1.2.3 Media used for bacterial cultures

Medium	Composition
LB-medium (Luria Bertani)	10 g Tryptone, 5 g Yeast extract, 10 g NaCl, dissolved in 1 l H_2O; autoclaved
LB- (Luria Betani) ampicillin medium	LB-medium + Ampicillin (50 mg/ml)
LB- (Luria Bertani) selection-agar-plates	LB-medium + 15 g Bacto™-Agar; autoclaved, cooled down to 37°C while stirring in water bath; addition of Ampicillin (50 mg/ml); poured into bacteria dishes and stored at 4°C until use

Table 4: List of all used bacterial media

3.1.2.4 Solutions for bacterial cultures

Solution	Composition
5-Bromo-4-chloro-3-indonyl-β-D-galactopyranoside (X-Gal)	20 mg X-Gal dissolved in 1 ml dimethylformamide; stored at -20°C
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	200 mg IPTG dissolved in 1 ml H_2O; stored at -20°C

Table 5: List of all used solutions for bacterial cultures

3.1.2.5 Solutions for molecular biological use

Solution	Composition
50x TAE buffer	40 mM Tris acetate, 2 mM EDTA; pH 8.0
TE buffer	50 mM Tris-HCl pH 8.3, 1 mM EDTA pH 8.0
6x DNA-loading buffer	0.25% (v/v) Bromphenolblue, 0.25% (v/v) Xylencyanol, 30% (v/v) Glycerol

Table 6: List of all used molecular biological solutions

3.1.2.6 Solutions for Immunocytochemistry

Solutions	Composition
Krebs-Ringer-Solution/0.1% BSA (KRH/A)	125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$ x $2H_2O$, 25 mM HEPES, 1.2 mM $MgSO_4$ x $7H_2O$, 1.2 mM KH_2PO_4, 5.6 mM Glucose, 0.1% BSA, pH 7.3
0.1 M Sodium phosphate buffer	0.1 M $NaHPO_4$ x $2H_2O$, 0.1 M NaH_2PO_4 x H_2O; pH 7.0; autoclaved
4% Paraformaldehyde	4 g Paraformaldehyde dissolved in 100 ml 0.1 M Sodium phosphate buffer at 60°C; stored -20°C
10x PBS	1.3 mM NaCl, 40 mM Na_2HPO_4, 14 mM KH_2PO_4, 24 mM KCl, pH 7.3
PBT1	0.1% Triton-X-100, 1% BSA dissolved in PBS
PBT2	0.1% Triton-X-100, 0.1% BSA dissolved in PBS; stored at -20°C

Table 7: List of all used solutions for immunocytochemistry

3.1.2.7 Antibodies used for immunocytochemistry

Antibody	Species	Dilution	Manufacturer
anti-Olig2	rabbit	1:500	Millipore
anti-p75	rabbit	1:600	Promega
anti-p75	mouse	1:200	Abcam
anti-Sox10	guinea pig	1:1000	Kindly provided by M. Wegner, University of Erlangen, Erlangen, Germany
anti-Sox10	goat	1:100	Santa Cruz
anti-Tuj1	mouse	1:500	HISS Diagnostic
anti-Tuj1	rabbit	1:500	HISS Diagnostic
anti-Peripherin	mouse	1:1000	Abcam
anti-O4	mouse	1:10	Hybridoma supernantant; hybridoma cells kindly provided by M. Schachner, Center for Molecular Neurobiology, Hamburg, Germany
anti-CH-PG 473HD	rat	1:100	Kindly provided by A. Faissner, Ruhr University Bochum, Bochum, Germany
anti-lex5750	rat	1:100	Kindly provided by A. Faissner, Ruhr University Bochum, Bochum, Germany
anti-Sox3	goat	1:500	R&D Systems
anti-Fabp7	rabbit	1:2000	Kindly provided by T. Müller, MDC, Berlin, Germany
anti-Tcfap2a	mouse	1:10	Hybridoma supernatant; Developmental Studies Hybridoma Bank
anti-Phox2b	rabbit	1:50	Kindly provided by C. Goridis, ENS, Paris, France
anti-CNPase	rabbit	1:100	Cell Signaling Technology
anti-GFAP	mouse	1:750	Sigma-Aldrich
Alexa 594 goat anti-mouse	goat	1:500	Life Technologies
Alexa 594 goat anti-rabbit	goat	1:500	Life Technologies
Alexa 546 goat anti-rat	goat	1:500	Life Technologies
Alexa 488 goat anti-mouse	goat	1:500	Life Technologies
Alexa 488 donkey anti-mouse	donkey	1:500	Life Technologies
Alexa 488 donkey anti-mouse	donkey	1:500	Life Technologies
Alexa 488 goat anti-rabbit	goat	1:500	Life Technologies
Alexa 488 goat anti-guina pig	goat	1:500	Life Technologies
Alexa Cy3 donkey anti-goat	donkey	1:500	Life Technologies
DAPI		1:500	Sanofis Aventis

Table 8: List of all used antibodies

3.1.3 DNA constructs and primers

3.1.3.1 Plasmids

Plasmid	Size of insert	manufacturer
pPB-CAG.Olig2-puDtk	931 bp	Marlen Weber
pPB-CAG.Nkx6.2-puDtk	1045 bp	Marlen Weber
pCMV5-GTXfl (=Nkx6.2)	1045 bp	Stephan Rehberg
pCMV5-Olig2	931 bp	Elisabeth Sock
pPB-CAG.OSKM-puDtk	5004 bp	Yusa et al., (2009)
mPB	1787 bp	Cadiñanos and Bradley, (2007)

Table 9: List of all plasmids

3.1.3.2 Primers for cloning

Name	Sequence (5´ → 3´)
Olig2-sense	ATGAATTCACCACCATGGACTCGGACGCCAG
Olig2-anti	ATGACGTCATGCATTTACTTGGCGTCGGAGGTG
Nkx6.2-sense	ATGAATTCGCGCCCATGGACGCTA
Nkx6.2-anti	ATATGCATTCACAAGGCGTCCCCC

Table 10: List of all primers used for cloning

3.1.3.3 Primers for RT-PCR

Name	Sequence forward	Sequence reverse	Annealing Temp. (°C)
Hoxb6	GGTTCAATGGTAGATTCGCTG	CTCCTCTTCTTCCTTCTAGGT	58
Sox1	CAGAGTCTCTCACCCAGGTCTTAT	TTGAGCTCTGGTCTTACTGAATTG	59
Tcfap2a	CACACACACCGGGGCTTA	GTACTTCCGCCACCGTGA	63
Tcfap2b	CCTGAAGTGTGGACATGAAATAAG	ACCTAGGAACAAACTCCATGAGAC	59
Dlx2	GTAGCCTCATTTCTTGTTTTCCTC	ACTATTCGGATTTCAGGCTCAAG	59
Bgn	TATGCTTCCTGACACTTTTCTTCC	CGTCTTGATAGCAGAGTATGAACC	59
Oct4	AGCCTTAAGAACATGTGTAAGCTG	ACTGAGTAGAGTGTGGTGAAGTGG	59
Nanog	ATCCCGAGAACTATTCTTGCTTAC	ATCAGACCATTGCTAGTCTTCAAC	59
Klf4	GGAGAAAGGAAGAGTTCAAGAGAC	ATTTTCCTTGTCAAAAGTATGCAG	59
Sox2	AGTGGTACGTTAGGCGCTTC	CGATATCAACCTGCATGGAC	59
Olig1	CAAAAGAGGAACAGCAGCAG	GTGGCAATCTTGGAGAGCTT	58
Olig2	CACAGGAGGGACTGTGTCCT	GGTGCTGGAGGAAGATGACT	58
Pax6	CGGAGGGAGTAAGCCAAGAG	TCTGTCTCGGATTTCCCAAG	58
Dlx1	CTACTCCATGCACTGTTTACACTC	AAGTACTGAGTTTGTTGGAACCTC	59
Erbb3	AGCAGAGGTATTAATGAGCAAACC	TAATGCAGACTGGAATCTTGATGG	59
Mgp	TGAAATCAGTCCCTTCATCAACAG	CTGTTTCTTCGCGCTAATATTTGG	59
Lgals3	CAAAATACAAGTCCTGGTTGAAGC	CACACAAAGTTTAAAAGGCTAGGG	59
Rgs5	TGCTAACTCTTTCGCTCATGTTAG	TTGAGAATGTCCAGATAACACAGC	59
Kcnj8	AAACCAGTGTCCATCAGAATCATG	CAGGACCTAAGAATGACGAAACTG	59
Pdgfrb	ATACTTACTACGTCTACAGCCTCC	CTCAGCAATTTCTACATCTCCCAG	59
S1pr3	TTCTGTATTCTCAAAAGCCTCACC	CTTGAACTCAGGACCATACAGATG	59
Abcc9	CACACACACATTTTCACAGCATAG	TTAAAATGAGTACAGGAACCACGG	59
Ly6a	CTCTGAGGATGGACACTTCT	GGTCTGCAGGAGGACTGAGC	59
Hoxc10	GAGCGCTATAACCGTAACGC	CTGAGGCGATTCCAGATGTT	58
Emx1	GAGCCTTTGAGAAGAATCAC	CTAGTCATTGGAGGTGACAT	58
Foxg1	TTTGCCATTTCATTCAAACCTGAC	GACCTGTTAGTGACCACATACATC	58
Nr2e1	ATGCCCCGTAGACAAGACAC	GGCCCATTGTGCTATTCCTA	58
Gapdh	ACTCAAGGGCATCTTGGGCTACAC	TGGGTGGTCCAGGGTTTCTTACTC	57

Table 11: List of all primers used for RT-PCR

3.1.4 Organisms

3.1.4.1 Mus musculus

Strain name	Origin
C57Bl/6J	Charles River Deutschland GmbH, Germany
Sox10-GFP	Kindly provided by Bill Richardson, UCL, London, UK (Kessaris et al., 2006)

Table 12: List of all used mouse lines

3.1.4.2 Bacteria

Strain name	Origin
ELECTROMAX™ DH5α-E™ competent cells	Invitrogen

Table 13: List of all used bacterium lines

3.2 Methods

3.2.1 Cell culture

Eukaryotic primary cells were treated under sterile conditions (laminar flow hood and sterile materials).

3.2.1.1 Tissue-dissection and dissociation to primary cells

Tissues were isolated from embryonic, postnatal or adult mice under semi sterile conditions. The dissociation of the tissues was performed under sterile conditions (laminar flow hood and sterile materials). The dissection and cell dissociation of the different tissues will be described below. For the centrifugation steps the HERAEUS MULTIFUGE 3S-R Centrifuge was used.

3.2.1.1.1 Dissection and dissociation of the dorsal root ganglia (DRG) from E12.5 mouse embryos

12.5 days pregnant C57Bl/6J mice were killed by cervical dislocation. The abdomen was opened and the uterus was removed to isolate E12.5 mouse embryos. The embryos were transferred to MEM (Invitrogen) and staged after Karl Theiler (Theiler, 1989). Afterwards the embryos were decapitated, the abdomen was opened and all inner organs were removed. The spinal column was opened laterally and the ventral part of the spinal column was removed. The spinal cord and appended DRGs were now accessible for the removal/dissection. DRGs were separately dissected, their processes were removed and DRGs were transferred into PBS/Glucose. DRGs were transferred into a 15 ml reaction tube (BD Falcon) and washed with PBS. Thereafter, DRGs were centrifuged for 2 min at 800 g. Afterwards, DRGs were incubated for digestion with a trypsin solution (trypsin 0.1% in PBS (w/v)) for 8 minutes at 37°C. To

stop the digestion, 1 ml PBS with soybean-trypsininhibitor and desoxyribonuclease 1 (DNase1) was supplemented. The DNase1 destroys released genomic DNA. A Single cell suspension was obtained by mechanical dissociation, using a fire polished siliconized pasteur-pipette. Subsequently, the cells were centrifuged for 2 min at 800 g, supernatant discarded and the appropriate medium was added (see Materials, Table 2). The cell number per ml was determined using the Neubauer Chamber.

3.2.1.1.2 Dissection and dissociation of the spinal cord (SC) from E12.5 mouse embryos

12.5 days pregnant C57Bl/6J mice were killed by cervical dislocation. The abdomen was opened and the uterus was removed to isolate E12.5 mouse embryos. The embryos were transferred to MEM (Invitrogen) and staged after Karl Theiler. Supsequently, the embryos were decapitated, the abdomen was opened and all inner organs were removed. The spinal column was opened laterally and the ventral part of the spinal column was removed.
The SC was dissected from the DRGs and the Meninges and were carefully removed. Afterwards, the spinal cord was collected in PBS/Glucose and cut into small pieces. The cell dissociation was performed as described in section 3.2.1.1.1.

3.2.1.1.3 Dissection and dissociation of the dorsal root ganglia (DRG) from P3 mouse

3 days old C57Bl/6J mice were killed by direct decapitation. The DRGs were dissected as described for embryonic derived DRGs (see section 3.2.1.1.1). DRGs were collected in PBS/Glucose and afterwards transferred into a 15 ml reaction tube (BD Falcon). They were washed with PBS, centrifuged for 3 minutes at 800 g and the supernatant was discarded. The cells were incubated for digestion with a trypsin solution (trypsin 0.1% in PBS (w/v)) for 50 minutes at 37°C. To stop the digestion, 1 ml PBS with soybean-

trypsininhibitor and desoxyribonuclease 1 (DNase1) was supplemented. A single cell suspension was obtained by mechanical dissociation, using a fire polished siliconized pasteur-pipette. Afterwards, the cells were centrifuged for 3 minutes at 800 g, the supernatant was removed and the appropriate medium was added (see Materials, Table 2). The cell number per ml was determined using the Neubauer Chamber.

3.2.1.1.4 Dissection and dissociation of the palate from adult mouse

The cells from adult palate were isolated from palatal tissue dissected from 3 to 5 C57Bl/6J mice or from *Sox10-GFP* mice (kindly provided by Bill Richardson, UCL, London, UK) of the age of 6 to 8 weeks, collected in PBS and cut into small pieces. The tissue was transferred into a 50 ml reaction tube (BD Falcon), washed with PBS and centrifuged for 10 min at 300 g. The supernatant was then removed. To obtain a single cell fraction, the tissue was treated with 250 U/ml collagenase (Worthington) for 3 h at 37°C. Afterwards, the cells were mechanically dissociated by pipetting and were washed through a 100 µm and 40 µm cell strainer (BD Falcon). The cells were centrifuged for 5 min at 400 g. The supernatant was discarded, fresh medium was added (see Materials, Table 2) and the cells were cultured as described below (see section 3.2.1.2.4).

3.2.1.2 Cultivation of primary cells

3.2.1.2.1 Coating of culture dishes

For the cultivation of primary cells and for the differentiation of neurospheres (NS), 4-well plates were coated with Poly-DL-ornithine laminin. At first Poly-DL-ornithine was dissolved in 0.15 M Borate buffer (0.5 mg/ml), applied to each well and incubated over night at RT (room temperature). Afterwards, the wells were washed twice with sterile H_2O and dried under a laminar flow hood. The 4-well plates were stored at 4°C. For

laminin coating, laminin was dissolved in PBS (10 µg/ml), applied to each Poly-DL-ornithine coated well and incubated for at least 2 hours at RT. Thereafter, the wells were washed three times with PBS and 80 µl medium was pipetted into each well.

3.2.1.2.2 Short-term cell cultures

Previously isolated cells were plated on poly-DL-ornithine laminin coated 4-well plates in standard differentiation medium without retinoic acid (RA) (see Materials, Table 2) and cultured for 3h at 37°C and 5% CO_2.

3.2.1.2.3 The generation and cultivation of neurospheres (NS) from E12.5 mouse DRGs (rNCSCs, BMP NCSCs and FGF NCSCs), P3 mouse DRGs (NCSCs derived from postnatal mouse DRGs) and E12.5 mouse spinal cord (SCSCs)

A NS is generated by a single stem cell, which produces daughter cells by continuous proliferation that leads to the emergence of a spherical cell aggregate. Neural stem cells generate neurospheres (NS) in vitro under specific medium conditions containing growth factors such as EGF and FGF (Reynolds and Weiss,1992; Reynolds and Rietze, 2005). A NS contains differentiated cells that are located inside of the NS as well as proliferating stem cells that populate the external layer. To avoid necrosis of cells inside the NS, due to an insufficient supply of nutrients, the NS have to be enzymatically and mechanically dissociated into single cells after reaching a certain size. Plated single cells are able to generate NS.

For the cultivation of rNCSCs, BMP NCSCs, FGF NCSCs, SCSCs and NCSCs derived from postnatal mouse DRG, cells of different tissues were cultured in a proliferation medium (see Materials, Table 2) on uncoated petri-dishes (3,5 cm, 6 cm from Becton Dickson and 10 cm petri dishes from SARSTEDT) at 37°C and 5% CO_2 for 5 to 10 days. After the generation of NS, NS and the medium were transferred into a 50 ml reaction tube (BD Falcon) and centrifuged for 5 min at 800 g. The supernatant (conditioned

medium) was not discarded because it contains unknown secreted factors from the NS that increases the efficiency of NS generation. Therefore, the conditioned medium was removed from the pellet and was sterile filtrated using a syringe with a 0,20 µm filter (VWR). A half of conditioned as well as a half of fresh proliferation medium were mixed for cultivation. The NS-pellet was transferred to 1 ml Accutase (PAA) and incubated for 30 to 45 min at 37°C. Afterwards, a single cell suspension was obtained by mechanical dissociation, using a fire polished siliconized pasteur-pipette. Thereafter, the cells were centrifuged for 5 min at 800 g, the supernatant was removed and the cells were washed through a 40 µm cell strainer (BD Falcon) to obtain a single cell fraction. The cell number per ml was determined by using the Neubauer Chamber. For the generation and propagation of secondary NS and all subsequent passages, cells were cultured in proliferation medium (a half fresh medium as well as a half conditioned medium) (see Materials, Table 2) on uncoated petri-dishes at 37°C and 5% CO_2.

For delayed BMP4 application, passage 3 rNCSCs were dissociated into single cells as described above and cultured in a proliferation medium which was used for BMP NCSCs (see Materials, Table 2). The cells were analyzed at passage 5.

For delayed BMP4 removal, passage 3 BMP NCSCs were dissociated into single cells as described above and cultured in a proliferation medium which was used for rNCSCs (see Materials, Table 2). The cells were analyzed at passage 5.

3.2.1.2.4 The generation and cultivation of NS from adult palate

The isolation of palatal cells was already described above (see section 3.2.1.1.4). For the generation and propagation of NS, cells from adult palate were cultured in cell culture flasks (greiner bio-one) containing 3 ml proliferation medium (see Materials, Table 2) at 5% CO_2, 5% O_2 and 37°C. The same amount of fresh medium was added every 3 to 4 days. After 7 days in culture, the NS were passaged. The NS including medium were transferred into a 15 ml reaction tube (BD Falcon) and centrifuged for 10 min at 150 g. Afterwards, the supernatant was discarded and 0.05% trypsin-EDTA was added

for enzymatically dissociation. The NS including trypsin were transferred into the used cell culture flasks and incubated in an incubator by shaking for 5 min at 37°C. Thereafter, the NS and trypsin-EDTA were transferred back into the 15 ml reaction tube and PBS was added. NS suspension was centrifuged for 10 min at 150 g. The supernatant was removed and a fresh proliferation was medium added. A single cell suspension was obtained by mechanical dissociation by pipetting. The cell number per ml was determined by using the Neubauer Chamber. For the generation of secondary NS and all subsequent passages, the cells were cultured in culture flasks containing 3 ml fresh proliferation medium (see Materials, Table 2) at 37°C, 5% O_2 and 5% CO_2.

3.2.1.2.5 NS short term cultures

To analyze the cell type identity of NS cells under proliferation conditions, NS at a defined passage were transferred to poly-DL-ornithine laminin coated 4-well plates. This results in the attachment of NS to the culture surface. In the presence of the proliferation medium (see Materials, Table 2) the NS were incubated for 24 h at 37°C and 5% CO_2 or for pNCSCs at 37°C, 5% O_2 and 5% CO_2. Afterwards, the attached NS cells were analyzed via immunocytochemistry.

3.2.1.2.6 NS differentiation

NS cells show the ability to differentiate into different cell types under differentiation conditions. To examine the differentiation potential of NS cells, NS at a defined passage were plated into poly-DL-ornithine/laminin coated 4-well plates and cultured as surface attached cells in the presence of a differentiation medium (see Materials, Table 2). NS were incubated for 7 days at 37°C and 5% CO_2. For neuronal differentiation of BMP NCSCs the incubation period requires 14 days. A medium change was performed every 3 to 4 days. Thereafter, differentiated NS were analyzed via immunocytochemistry.

3.2.1.2.7 Transfection of BMP NCSCs via electroporation

For the transfection of *mmOlig2* and *mmNkx6.2* into BMP NCSCs, the *Piggy Bac* (*PB*) transposon system was used. *PB* is originally isolated from *Trichoplusia ni* (Cary et al., 1989). The *PB* transposon system is based on a cut and paste mechanism. The *PB* transposon vector contains two inverted terminal repeat sequences (ITRs) at both ends of the inserted gene. The *PB* Transposase recognizes the ITRs, cuts the insert at the ITRs and integrates it into TTAA chromosomal sites (Ding et al., 2005). The advantage of the *PB* transposon system is that the inserted gene can be removed from the genome by introducing a *PB* Transposase without leaving footprints (Mitra et al., 2008). Transient transgene introduction via *PB* transposon is expected to avoid permanent genetic alteration of cells in contrast to the retrovirally introduced genes.

In this study a two-plasmid donorhelper system was used. The donor plasmid is carrying the gene of interest plus ITRs. In the present study the donor plasmid, pPB-CAG.OSKM-puDtk, was used (Yusa et al., 2009). OSKM was replaced either with *mmOlig2* (pPB-CAG.Olig2-puDtk) or *mmNkx6.2* (pPB-CAG.Nkx6.2-puDtk). The helper plasmid (mPB) is carrying the transposase that catalyzes the insertion of the gene of interest into the host genome (Cadiñanos and Bradley, 2007).

For the transfection of passage 1 BMP NCSCs via electroporation the small cell number kit (SCN) (Lonza AG) was used. Primary BMP NCSCs were dissociated into single cells (see section 3.2.1.2.3). 6000 cells were centrifuged at 800 g for 5 minutes and the supernatant was discarded. 20 µl Nucleofector™, containing *PB* donor plasmid with *mmOlig2* (pPB-CAG.Olig2-puDtk) or *mmNkx6.2* (pPB-CAG.Nkx6.2-puDtk) at DNA a concentration of 1,30 µg as well as the helper plasmid (mPB) at a DNA concentration of 0,64 µg, was added to the cells and transferred to a cuvette. The cells were electroporated by using the SCN program 2 (Amaxa Biosystems). Afterwards, 500 µl proliferation medium, used for the cultivation of rNCSCs (see Materials, Table 2), was added to each cuvette. For a step gradient density centrifugation, to remove cellular debris, 3 ml DMEM/F12 (Sigma-Aldrich) containing 3% BSA (high density)

was filled in a 15 ml reaction tube (BD Falcon) and overlayed with the cell suspension. Thereafter, the cells were centrifuged at 60 g for 25 minutes, the supernatant containing all debris was discarded and the proliferation medium was added. The cell number per ml was determined by using the Neubauer Chamber. Transfected cells were plated with proliferation medium that was used for rNCSCs (see Materials, Table 2) on poly-DL-ornithine laminin coated 4-well plates for 16 h to analyze the transfection efficiency or in a medium containing half fresh proliferation medium and half conditioned medium of rNCSCs (see Materials, Table 2) on petri-dishes or poly-DL-ornithine laminin coated 4-well plates for the propagation and generation of NS.

3.2.2 Immunocytochemistry

3.2.2.1 Fixation of cultured cells

The cells were washed twice with 1 ml Krebs-Ringer-solution/0.1% BSA, fixed with 1 ml 4% paraformaldehyde in 0.1 M NaH_2PO_4 pH 7,3 for 15 minutes and washed twice with 1ml PBS (phosphate-buffered saline). The cells can be stored at 4°C for at least 3 weeks.

3.2.2.2 Immunostainings of intracellular antigens

After blocking with PBT1 (1% BSA/0.1% TritonX100/PBS) for at least 30 minutes, 60 µl of PBT1 containing the diluted antibody (see Materials, Table 2) was applied to the cells. The cells were incubated with primary antibodies in PBT1 for 1 hour. Afterwards, the cells were washed twice with 80 µl PBT2 (0.1% BSA/0.1% TritonX100/PBS) and incubated with secondary antibodies and DAPI in 60 µl PBT2 for one hour. After washing the cells with 80 µl PBT2 and 80 µl PBS the cells were mounted with Poly-Aquamount and glass coverslips.

The staining for mouse anti-Tcfap2a and mouse anti-p75 involved overnight incubation

with primary antibodies at 4°C. Mouse anti-p75 was applied in PBS rather than in PBT1. The second antibody staining was performed as described above.

3.2.2.3 Cell surface immunostainings

To detect O4, 473HD and Lex5750 epitopes, cells were washed twice with Krebs-Ringer-solution/0.1% BSA followed by the incubation with the primary antibodies in 60 µl Krebs-Ringer-solution/0.1% BSA for 20 minutes (O4) or 40 minutes (Lex473, Lex5750). The cells were fixed afterwards and secondary the antibody staining was performed as described above (see section 3.2.2.1 and 3.2.2.2).

3.2.2.4 Quantification of immunostainings

Immunostainings were analyzed at a fluorescent microscope (Carl Zeiss Microimaging GmbH, Jena, Deutschland)

3.2.3 Working with nucleic acids

3.2.3.1 RNA isolation

Total RNA was isolated from the mouse 46C ES cell line (kindly provided by A. Smith, Stem Cell Institute, University of Cambridge, UK), passage 3 rNCSCs, BMP NCSCs, SCSCs and pNCSCs using the RNeasy Kit and QIAshredder (Qiagen), following manufacturers instructions (Qiagen).

3.2.3.2 cDNA synthesis

cDNA from RNA was synthesized using the M-MLV Reverse Trancriptase Kit (Invitrogen), following manufacturers instructions (Invitrogen).

3.2.3.3 PCR (Polymerase Chain reaction)

Selective DNA sequences can be amplified by PCR over a billion times.

The reaction solution was composed by following the manufacturers instructions (Invitrogen). For the DNA synthesis of *mmOlig2* the reaction solution contains 6% DMSO. The DNA-polymerase used in this work is derived from the heat-stable microorganism *Thermus aquaticus* (Taq) (Invitrogen, Karlsruhe, Germany).

PCR procedure:

1. Initiation step: At this step the reaction must be started at 95°C, which is followed by the addition of the Taq-DNA polymerase (manual hot start taq) which needs a heat step to get activated.
2. Denaturation step: the primer and the template DNA were denaturated at 95°C.
3. Annealing step: The reaction temperature was lowered to a primer dependent temperature to allow the annealing of the primer and DNA single strands.
4. Elongation step: This reaction needs a temperature at 72°C. At this time the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand.

Step 2 to 4 are repeated in a desired number. The PCR products are analyzed by gel electrophoresis (see section 3.2.3.4).

For the DNA sythesis of *mmNkx6.2* the HotSarTaq® Plus Master Mix Kit (Qiagen, Hilden, Germany) was used. The DNA-polymerase was directly added to the the reaction solution. The reaction solution was composed, following the manufacturers instructions (Qiagen, Hilden, Germany). The PCR procedure was performed as described above.

The Real time PCR (RT-PCR) was performed using the HotStarTaq® Plus Master Mix Kit (Qiagen), following the manufacturers instructions (Qiagen), and 160 ng of the chosen cDNA as template was added. The PCR procedure was performed as described

above. Samples were taken for all tissues 15 cycles after Gapdh detection, except of Gapdh taken 8 cycles after Gapdh detection.

3.2.3.4 Gel electrophoresis

The gel electrophoresis is a method to separate DNA strands by their length. To asses the length, a DNA ladder was used. Long fibers of agarose polymers were netted in a gel. The higher the concentration of agarose gel, the smaller the pores in the gel are. The gel electrophoresis functions as a filter, which is connected with an electrical field. The electrical field pushes the negatively charged DNA through the gel pores and separates DNA fragments according to their lengths. Smaller DNA fragments move faster through the gel pores than longer ones.
In order to prepare the agarose gel 1% - 3% (w/v), the agarose was dissolved in TAE. This mixture was boiled in a microwave until the agarose was melted. After the gel was hardened, the gel tray was transferred into a buffer chamber that contains TAE. The DNA solution was mixed in a ratio of 1:6 with a 6x loading buffer. The slots of the gel were filled with DNA solution by pipetting. Additionally, a 1 kb plus ladder (Invitrogen, Karlsruhe, Germany), which contains DNA fragments of known size, was pipetted in one slot. After the gel was loaded with DNA, it was run at 80-120 V and 40 mA. To detect the DNA, the gel was transferred into a bath containing Ethidium bromide for 30 minutes. Ethidium bromide intercalates with the DNA. The DNA can be visualized by using ultraviolet light.

3.2.3.5 Gel extraction

For the extraction of DNA from the gel, DNA bands were cut by a scalpel out of the gel. The extraction was performed, following the manufacturers instructions, using the QIAquik Gel Extraction kit (Qiagen, Hilden, Germany).

3.2.3.6 Measurement of DNA concentration

To measure DNA concentrations, a spectrophotometer (ND-1000) was used. DNA has a absorbance between 230 nm and 320 nm.

3.2.3.7 Ligation of DNA fragments

Ligation is a mechanism to integrate a double stranded DNA fragment into DNA strands that have double-strand breaks. The DNA ligase forms covalent phosphodiester bonds between the 3´hydroxyl end of one nucleotide with one 5´phosphate end of another. For the ligation into the pGEM®-T-Easy vector, pGEM®-T-Easy vector system (Promega) was used (Promega, Mannheim, Germany). For the ligation of DNA fragments into the *Piggy Bac* Donor plasmid the T4 DNA ligase (Invitrogen) was used. The ligation was carried out at RT for 2 h, followed by an overnight incubation at 4°C.

3.2.3.8 Transformation of electro-competent bacteria

The plasmid containing the insert can be transformed into the *E.coli* (*Escherichia coli*) bacteria strains by electroporation. For this, a 50 µl of DH5α bacteria solution was defrosted on ice and 1 µl of the DNA was added to the bacteria solution. The solution was transferred into a pre-cooled 2 mm electroporation cuvette (Eurogentec, San Diego, California, USA) und put into a gene pulser (Bio RAD). The gene pulser was set to 2.5 V (field strength 12 kV/m), the pulse controller to 200 Ω as well as the Capacitance Extender to 25 µFd. This allows a 5 ms pulse. The pulse leads to the emergence of little pores in the bacteria membrane which allows the DNA to enter. After the transformation, 200 µl LB-medium was added to the bacteria. The solution was transferred into a 15 ml reaction tube (BD Falcon) and incubated for 30 to 50 minutes at 37°C while shaking. Afterwards, the mix was plated on a LB-selection agar plate and incubated over night at 37°C. The agar plates were treated with ampicillin, X-Gal and IPTG before

it was plated for white blue screening

A white blue screening was used to detect a successful transformation of the pGEM®-T-Easy vector. The vector encodes a α subunit of the *LacZ* gene, which is transformed into E.coli. E.coli has an integrated lac operon in his host DNA which encodes the β-Galactosidase. IPTG is needed to induce the expression from the Lac Promotor. The α subunit of lacZ will disrupt the production of β-Galactosidase. X-Gal is required for the screen, which will be metabolized by β-Galactosidase to form an insoluble product which is bright blue. This functions as an indicator because cells which turn blue do not have the inserted gene in the plasmid. In turn, the cells which keep white have the inserted gene in the plasmid. White clones were picked one day after and afterwards transferred into Liquid LB-medium, containing ampicillin, and incubated at 37°C and 200 rpm shaking over night.

3.2.3.9 The isolation of Plasmid DNA from bacteria cultures

This method is used to extract the plasmid out of E.coli. 2ml of E.coli in media was taken and centrifuged. The cells were in the pellet. The supernatant was discarded. For the isolation of the plasmid the Qiaprep® Spin Miniprep kit (Qiagen) was used. For the extraction of higher DNA amounts the Qiagen ® Plasmid Midi kit (Qiagen) was used.

3.2.3.10 Digestion of DNA

The restriction enzymes or endonucleases are enzymes which are isolated out of the bacterias and cut specific DNA sequences, the so called restriction sites. The restriction enzymes mostly bind as homodimers to symetric DNA. Some enzymes as EcoR1 recognize continuous sequences and other recognize discontinuous sequences as Bgl2.

2 to 5 units restriction enzyme were added to 1 µl DNA and the according digestion

buffer (Roche, Darmstadt, Germany). The reaction was incubated 1 to 5 hours at 37°C. The analysis of the digestion was performed, using gel electrophoresis (see section 3.2.3.4).

3.2.3.11 Microarray analysis

Microarry analysis is used to quantify the expression level of ten thousands of genes within a tissue sample. In this study the Affymetrix microarray was used. One Affymetrix gene chip consists of 10 to 20 oligonucleotide pairs with a length of 25 bp that hybridize to the RNA of the transcribed gene. Each oligonucleotide pair consists of a perfect match and mismatch oligonucleotide. The perfect match oligonucleotide is complementary to the target RNA. The mismatch oligonucleotide has the identical sequence of the perfect match oligonucleotide except of one base, and is supposed to detect non-specific hybridization (reviewed in Hochreiter et al., 2006)

To perform a affymetrix microarry 3 replicates of each cultured 3rd passage rNCSCs, BMP NCSCs, SCSCs and pNCSCs, were transferred to TRIzol (Life Technologies) and shreddered twice by using the QIAshredder™ (Qiagen). The Cells which were lyzed in TRIzol were stored at -80°C until the RNA isolation was performed.

RNA isolation and Microarray analyses were performed by Dr. Galina Apostolova (Innsbruck Medical University, Institute for Neuroscience, Innsbruck, Austria), and as described in Weber et al., (2015): Total RNA was isolated using TRIzol reagent (Life Technologies), purified by RNeasy MinElute kit (Quiagen) and analyzed for RNA integrity (2100 Bioanalyzer). RNA samples from three independent cell cultures of rNCSCs, BMP NCSCs, SCSCs and pNCSCs were labeled and hybridized to Affymetrix Mouse Genome 430 2.0 Arrays. Microarray hybridization was performed at the Expression Profiling Unit of the Medical University Innsbruck. Pre-processing and differential expression analysis were performed in R (http://www.r-project.org) using packages from the Bioconductor project (Gentleman et al., 2004). Raw expression values were normalized and summarized using the GCRMA method (Wu et al., 2004).

3. Materials and Methods

The moderated t-test (Smyth et al., 2004) was applied to assess the significance of differential expression between the compared groups; the resulting p-values were adjusted for multiple hypothesis testing following the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995) for a strong control of false discovery rate. A gene was considered as differentially expressed when it was up- or down-regulated at least 1.5-fold and its adjusted p-value was lower than 0.05. Gene ontology analysis was carried out to study the biological function of genes differentially expressed between rNCSCs, BMP NCSCs and pNCSCs using DAVID (Huang et al., 2009). Principal component analysis (PCA) was performed on samples based on normalized expression of all genes. To correct for batch effects either ComBat package was used (Johnson and Li, 2007) or SCAN - a single-sample normalization approach which allows concurrent use of independent gene expression datasets (Piccolo et al., 2012). Profiling results have been deposited at the Gene Expression Omnibus (GEO) under accession GSE57003. The following publicly available expression data downloaded from GEO were used for comparison with the microarray data generated in this study: GSE50824 (GSM1230380, GSM1230381, GSM1230382, GSM1230383); GSE8034 (GSM200043, GSM200044, GSM200045).

(Weber et al., 2015)

4. Results

4.1 Detailed analysis of the reprogramming of DRG-derived NCSCs (neural crest-derived stem cells) to cells with CNS identity

Until now there are only few reports showing that NCSCs, derived from different post-migratory neural crest-derived tissues, are able to generate CNS cell types. However, these findings are of considerable interest as they suggest that NCSCs are a potential source for regenerative medicine in the CNS. The group of Baron-Van-Evercooren revealed that boundary cap NCSCs generate CNS oligodendrocyte-like cells in vitro and in vivo (Zujovic et al., 2011).

We demonstrated that Sox10-positive NCSCs from E12.5 DRG mouse embryos generate neurospheres (NS) and show the potential to self-renew. 3rd passage NS of DRG-derived NCSCs express the CNS transcription factor Olig2 and differentiate into CNS cell types, including neurons, oligodendrocyte-like and astrocyte-like cells and lack neural crest markers, such as p75 and Peripherin. Furthermore, in vivo experiments showed that the majority of injected DRG-derived NCSCs differentiate into cells of the oligodendrocyte lineage and are able to myelinate axons in brains of postnatal *shiverer* mice. The results indicated that NCSCs were reprogrammed to cells with CNS fates without genetic modification (Binder et al., 2011).

In the first part of this study the reprogramming of embryonic DRG-derived NCSCs (rNCSCs) towards CNS fates is analyzed in more detail.

4.1.1 3rd passage rNCSCs NS express several oligodendrocyte marker genes

Previous studies revealed that 3rd passage rNCSCs differentiate into oligodendrocyte-like cells in vitro and in vivo (Binder et al., 2011). While in vivo generated oligodendrocyte progeny were analyzed in great detail, in vitro generated oligodendrocyte-like cells were only identified by the coexpression of O4 and Olig2, the characteristic

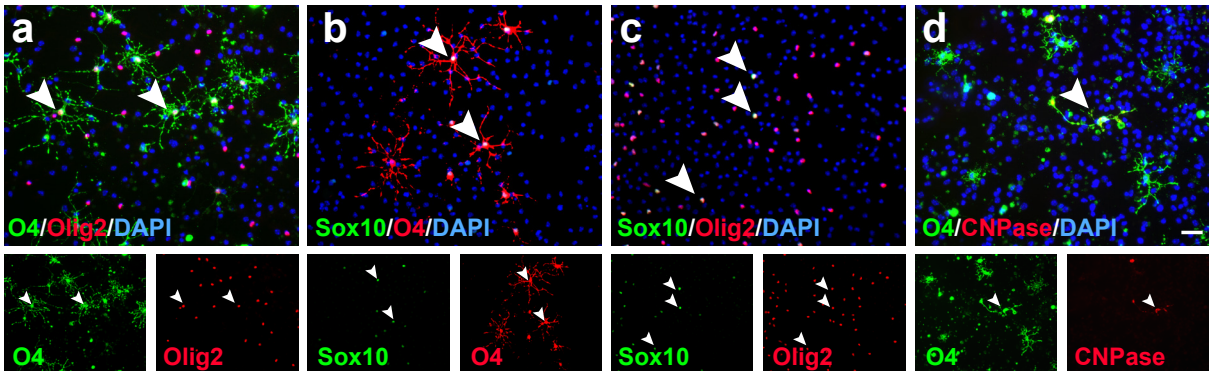

Figure 8: rNCSCs differentiate into oligendrocyte-like cells. 3rd passage rNCSCs differentiate into oligodendrocyte-like cells characterized by their morphology and the coexpression of O4/Olig2 (a), O4/Sox10 (b), Olig2/Sox10 (c) and O4/CNPase (d), see arrowheads. Scale bar: 30 µm. (Weber et al., 2015).

morphology of oligodendrocytes (Figure 8a) and the lack of the neural crest/neural crest-derivative marker p75 (Binder et al., 2011; Marlen Weber, Diploma thesis, 2010). Therefore, in vitro generated oligodendrocyte progeny of 3rd passage rNCSCs were analyzed for additional marker genes via immunocytochemistry upon treatment with standard differentiation medium (see Materials and Methods, section 3.2.1.2.6 and Table 2).

Identified O4-positive oligodendrocyte-like cells coexpress Sox10 (Figure 8b), a marker expressed in the oligodendrocyte lineage but also in Schwann cells and migrating neural crest cells (Britsch et al., 2001; Schreiner et al., 2007; Fu et al., 2002; Zhou et al., 2001). Sox10 is coexpressed with Olig2 (Figure 8c) and the quantification revealed that virtually all Sox10-positive cells coexpress Olig2 (Supplementary Figure 1). Olig2 is a marker gene known to be expressed in cells of the oligodendrocyte lineage and is not found in neural crest/neural crest derivatives, including Schwann cells of the the PNS (Vallstedt et al., 2005; Fu et al., 2002; Küspert et al., 2011; Binder et al., 2011). Additionally, CNPase-expressing cells were detected representing a small fraction of O4-positive cells (Figure 8d). CNPase is expressed in early myelinating oligodendrocytes (Sprinkle et al., 1989; Gravel et al., 1996; reviewed in Nicolay et al., 2007). Oligodendrocyte progeny were not further characterized via other myelin markers, such as MAG, MOG and MBP. The coexpression of O4/Olig2, O4/Sox10, Olig2/Sox10 and O4/CNPase and the lack of p75 (Binder et al., 2011; Marlen Weber,

Diploma thesis, 2010) strongly support the assumption that oligodendrocytes rather than PNS Schwann cells are generated from rNCSC in vitro.

4.1.2 Marker gene expression of rNCSCs is similar to spinal cord-derived stem cells (SCSCs)

Previous studies revealed that 3rd and 10th passage rNCSCs differentiate into CNS cell types, such as neurons, astrocyte-like and oligodendrocyte-like cells (Binder et al., 2011; Marlen Weber, Diploma thesis, 2010). In neurosphere cultures of CNS stem cells EGF and FGF induce Olig2 expression in virtually all NS cells (Hack et al., 2004). Olig2 expression was also detected in 3rd and 10th passage rNCSCs by qPCR under proliferation conditions (Binder et al., 2011). However, upon differentiation only a subpopulation of cells (17,9%) express Olig2 (Marlen Weber, Diploma thesis, 2010). Thus, it was unknown if also under proliferation conditions only a fraction of rNCSCs express Olig2. It was also unclear if p75 expression is lost during differentiation or under proliferation conditions. Furthermore, it was of interest to examine if rNCSCs contain differentiated cell types.

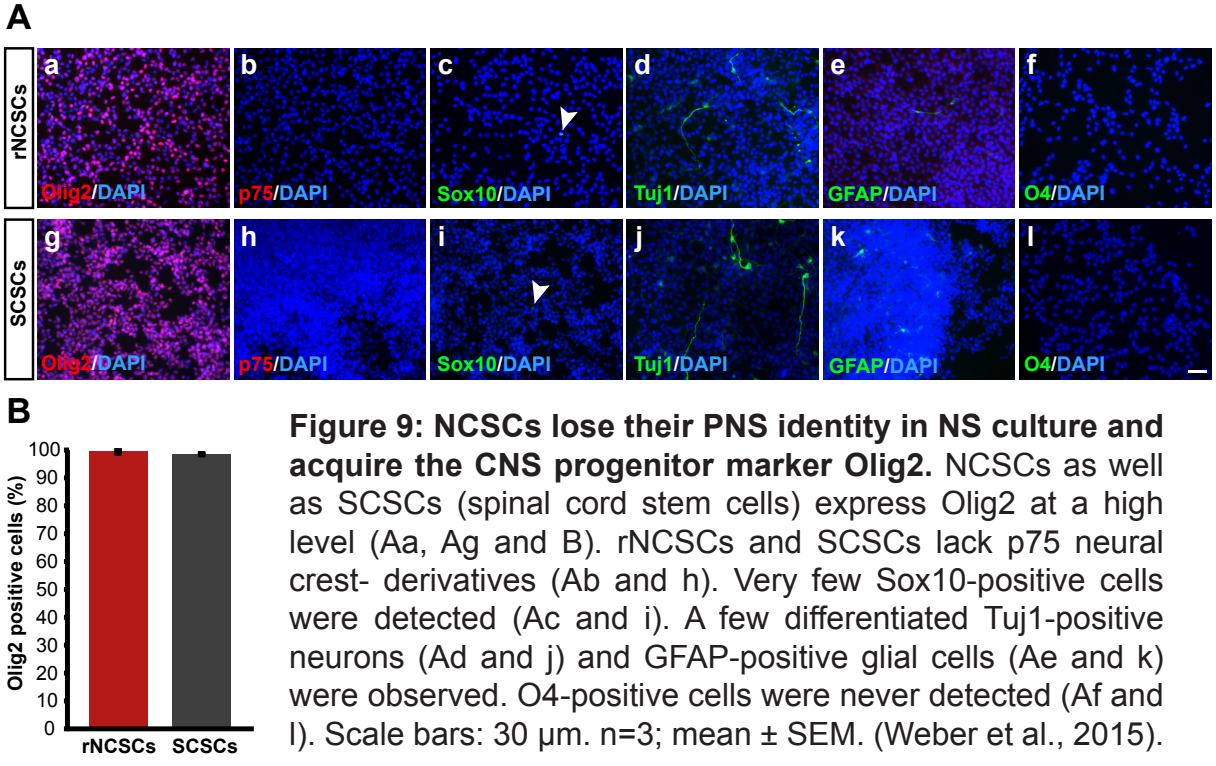

Figure 9: NCSCs lose their PNS identity in NS culture and acquire the CNS progenitor marker Olig2. NCSCs as well as SCSCs (spinal cord stem cells) express Olig2 at a high level (Aa, Ag and B). rNCSCs and SCSCs lack p75 neural crest- derivatives (Ab and h). Very few Sox10-positive cells were detected (Ac and i). A few differentiated Tuj1-positive neurons (Ad and j) and GFAP-positive glial cells (Ae and k) were observed. O4-positive cells were never detected (Af and l). Scale bars: 30 µm. n=3; mean ± SEM. (Weber et al., 2015).

For the analysis short-term cultures of 3rd passage rNCSCs were used (see Materials and Methods, Section 3.2.1.2.5 and Table 2). The quantification of immunostainings indicated that virtually all cells of rNCSCs NS express Olig2 under proliferation conditions (Figure 9Aa and B). rNCSCs NS lack p75-positive cells (Figure 9Ab) and O4-expressing glial cells (Figure 9Af). Sox10-positive cells (Figure 9Ac), Tuj1-positive neurons (Figure 9Ad) and GFAP-positive glial cells (Figure 9Ae) were only detected in very low numbers. Of note, similar results were obtained from 3rd passage spinal cord stem cells (SCSCs). SCSCs were isolated from E12.5 spinal cord and treated as described for rNCSCs (see Materials and Methods, section 3.2.1.1.2, 3.2.1.2.3, 3.2.1.2.5 and Table 2). Olig2 expression was detected in virtually all SCSCs, shown by quantification (Figure 9Ag and B). SCSCs lack p75 (Figure 9Ah) and O4 expression (Figure 9Al). A few Tuj1- (Figure 9Aj), GFAP- (Figure 9Ak) and Sox10-positive cells (Figure 9Ai) were detectable in SCSCs. This data set confirms that rNCSCs in NS culture acquire CNS marker gene expression. Additionally, the same marker genes are expressed by SCSCs, suggesting that rNCSCs have acquired a CNS identity during NS culture.

4.1.3 The reprogramming of DRG NCSCs (rNCSCs) is complete at passage 2 to 3 and is maintained at least up to passage 10

It was unknown at which passage the reprogramming of rNCSCs to cells with CNS identity starts and when the reprogramming is completed. Therefore, short-term DRG cultures and 1st, 2nd, 3rd and 10th passage rNCSCs were analyzed for the proportion of p75- and Olig2-positive cells (see Materials and Methods, section 3.2.1.2.5). The quantification indicated that p75, a marker gene for neural crest and neural crest derivatives (Morrisson et al., 1999; Paratore et al., 2001), is expressed in a high proportion of cells in the E12.5 DRG starting population (96.5% ± 0.5%, n=3 (Figure 10A)) and is strongly decreased in passage 1 (P1) NS (7.8% ± 5.9%, n=4 (Figure 10A)) and lost between passage 2 and 3 (P2: 0.1% ± 0.0%, P3: 0.0%, n=4 (Figure 10A)).

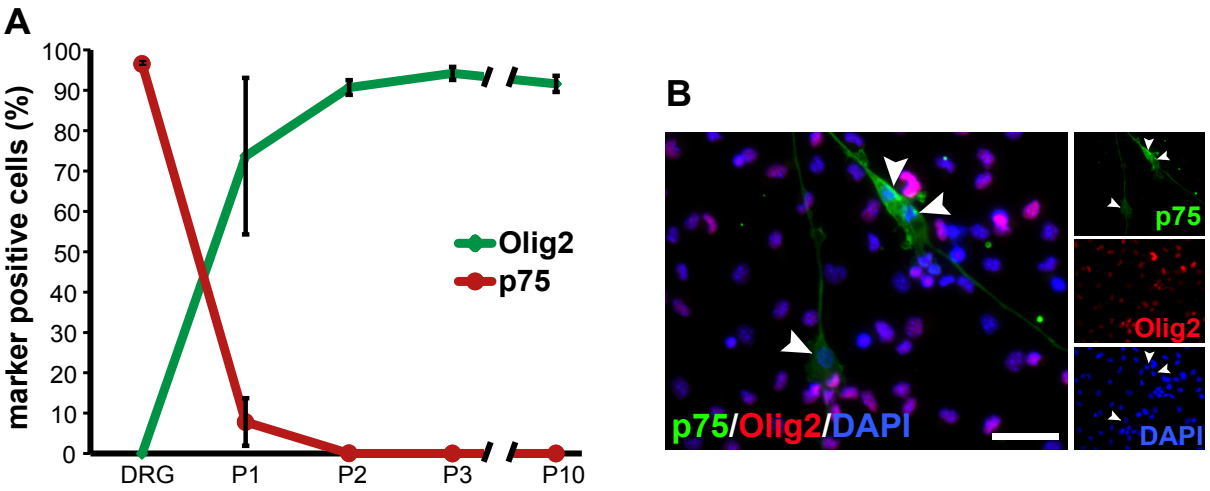

Figure 10: p75 is downregulated and Olig2 is upregulated in rNCSCs during long term culture. (A) The proportion of p75- and Olig2-positive cells was analyzed in DRG short-term cultures and first, 2nd, 3rd and 10th passage (1-10P) NS via immunocyto-chemistry. p75 is downregulated (DRG starting population: 96.5% ± 0,5%, P1: 7.8% ± 5.9%, P2: 0.1% ± 0.0% (n=3 to 4; mean ± SEM)) and is undetectable from passage 3 onwards. Olig2 is not detected in DRG cultures and is upregulated (P1: 73.7% ± 19.4%, P2: 90.7% ± 1.8%, P3: 94.2% ± 1.6% and P10: 89.7% ± 2.0% (n=3 to 4; mean ± SEM)). (B) Olig2/p75-coexpressing cells were never detected in first passage NS. Scale bar : 30 µm. (Weber et al., 2015).

The CNS marker gene Olig2 is undetectable in the starting population of E12.5 DRGs (n=5) (Figure 10A). As Olig2 is expressed in E12.5 spinal cord (Fu et al., 2002; Zhou et al., 2001) a contamination of the DRG preparation by CNS cells from the spinal cord is excluded. Of note, Olig2 expression appeared in the majority of cells already in passage 1 NS cells (P1: 73.7% ± 19.4%, n=4 (Figure 10A)), the first time point analyzed. The number of Olig2-expressing cells peaks at passage 2 to 3, where more than 90% of the NS cells express Olig2 (n=4 (Figure 10A)). Furthermore, a homogeneous population of Olig2-positive and p75-negative cells is at least maintained until passage 10 (Olig2: 89.7% ± 2.0%, p75: 0.0 ± 0.0 (n=3)) (Figure 10A), the latest passage analyzed. p75- and Olig2-coexpressing cells were never observed in passage 1 NS cells (Figure 10B). This leads to the assumption that p75 is downregulated before Olig2 is expressed in rNCSCs. The results demonstrate that NCSCs derived from E12.5 DRG acquire the CNS marker Olig2 already in passage 1 and lose their PNS identity, indicated by the loss of p75.

4.1.4 rNCSCs acquire a SCSC identity in NS culture

Since the expression of the marker genes, Olig2, p75, Sox10, Tuj1, GFAP and O4, seemed to be identical in 3rd passage rNCSCs and SCSCs (see section 4.1.2), it was of interest to examine if rNCSCs express further CNS (progenitor) marker genes. To address this question gene expression profiles of 3rd passage rNCSCs and SCSCs were compared by Affymetrix microarrays (see Materials and Methods, section 3.2.3.11). Interestingly, rNCSCs and SCSCs revealed a virtually identical gene expression pattern (Figure 11A). Among the 45.101 probe sets on the array, only one gene (Efcab7) was differentially expressed following the established criteria for differential expression (adjusted p-value < 0.05; fold change ≥ 1.5). The scatter plot of microarray data demonstrates that rNCSCs and SCSCs express genes at a high level that are expressed in the spinal cord neuroepithelial cells and ventricular zone cells, such as Fabp7, Ptprz1, Tnc, Olig1, Olig2, Pax6, Sox1, Sox2, and Sox3 (Figure 11A) (Feng et al., 1994; Matsumata et al., 2012; Arai et al., 2005; Ligon et al., 2007; Lu et al., 2002; Sun et al., 2003; Fu et al., 2002; Meijer et al., 2014; reviewed in Thiel 2006; Garcion et al., 2001; Ito et al., 2005; Sirko et al., 2010; Karus et al., 2013; reviewed in Sarkar and Hochedlinger, 2012). However, genes expressed in neural crest/neural crest derivatives and mesenchymal neural crest derivatives, such as Snai2, Tcfap2a, Dlx1, Dlx2, Erbb3, Phox2b, Mgp, Lgals3 and Bgn (Sakai et al., 2005; Wolpwitz et al., 2000; Morrissey et al., 1995; Erickson et al., 1997; reviewed in Hilger-Eversheim, 2000; Schorle et al., 1996; Zhang et al., 1996; Moser et al., 1997; reviewed in Rohrer et al., 2011; Thomas et al., 1997; Lou et al., 1997; Wilda et al., 2000; Yu et al., 2013; Henderson et al, 2008; Hsu et al., 2000) are undetectable or expressed at background levels (Figure 11A). For a number of genes indicated in the scatter plot the differential expression was confirmed via RT-PCR (Figure 11B) and/or immunocytochemistry (Figure 11C) (indicated in red in the scatter plot). The immunocytochemistry analyses showed a virtually uniform expression of Sox3, Fabp7, the chondroitin sulphate proteoglycan CH-PG (recognized by the mAB 473HD) and the LewisX (LeX) glycan

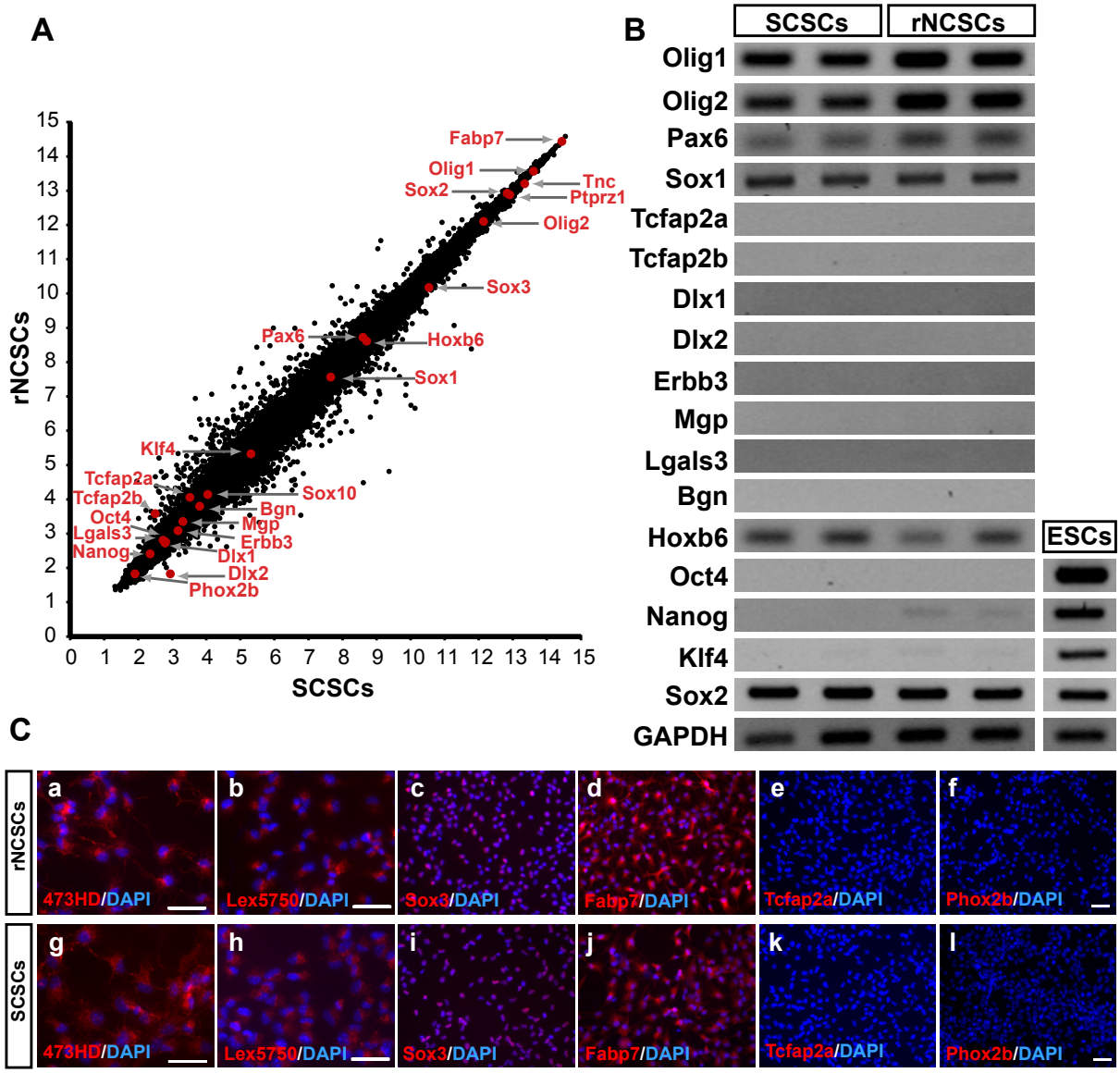

Figure 11: rNCSCs show uniform expression of CNS marker genes. 3rd passage rNCSCs and SCSCs were analyzed via affymetrix gene array (A), RT-PCR (B) and immunocytochemistry (C). The scatter plot shows a virtual identical gene expression pattern of rNCSCs and SCSCs (A). Genes characteristic for CNS stem cells, neural crest and neural crest derivatives as well as ESCs genes are indicated (red). Differential expression represents *log2* fold change. (B) RT-PCR confirms gene array data (CNS marker: Olig1, Olig2, Pax6 and Sox1; Neural crest and cranial mesenchymal neural crest marker: Tcfap2a, Tcfap2b, Dlx1, Dlx2, Erbb3, Mgp, Lgals3 and Bgn; Pluripotency marker: Oct4, Nanog, Klf4 and Sox2). Trunk anteroposterior (AP) identity is shown by Hoxb6 expression (B). (C) Virtually all cells express 473HD, Lex5750, Sox3, Fabp7 and are devoid of Tcfap2a and Phox2b. Scale bars: 30 µm. (Weber et al., et al. 2015).

(recognized by the mABs 5750^{Lex}) (Figure 11C). Tnc and Ptprz1 are carriers of the CH-PG and LeX glycan, are expressed during development of the mouse spinal cord and are important compounds of the CNS stem cell niche (Karus et al., 2013; Sirko et al., 2007; Capela et al., 2006; Sirko et al., 2010).

In order to analyze if rNCSCs undergo a pluripotency state during reprogramming, RT-PCRs were performed to characterize pluripotency gene expression, such as Nanog, Klf4, Oct4 and Sox2 in rNCSCs as compared to embryonic stem cells (ESCs). rNCSCs and SCSCs express Nanog, Klf4 and Oct4 at background levels and Oct4 expression was undetectable (Figure 11A and B). Sox2 is expressed at high levels in rNCSCs and SCSCs (Figure 11A and B) as expected from Sox2 expression in the developing spinal cord, ventricular zone stem cells and glial lineage (Aquino et al., 2006; Le et al., 2005; Cavallaro et al., 2008; Ferri et al., 2004; Graham et al., 2003; Miyagi et al., 2008; Cimadamore et al., 2011). Furthermore, rNCSCs show low level expression of pluripotency genes even at passage 1 (Supplementary Figure 3), the time point when rNCSCs switch from PNS to CNS identity (see section 4.1.3). This suggests that reprogramming does not involve a transient pluripotency state. The anteroposterior (AP) identity of rNCSCs and SCSCs is maintained, as shown by the expression of Hoxb6 and Hoxc10 which are absent in cranial tissues such as E14.5 fore/midbrain (Figure 11B and 12). Conversely, fore/midbrain markers, such as Emx1, Foxg1 and Nr2e1 (Kelly et al., 2009; Bishop et al., 2001) were only detectable at background levels or were even lacking in rNCSCs, SCSCs and E14.5 spinal cord

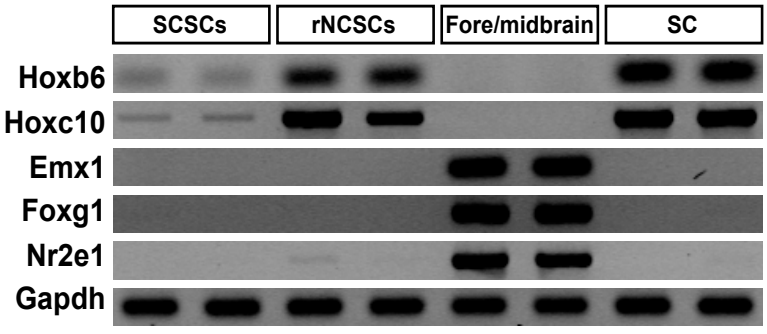

Figure 12: The anteroposterior identity is maintained by DRG-derived rNCSC neurospheres. RT-PCR of rNCSCs, SCSCs, E14.5 fore/midbrain and E14.5 Spinal cord (SC) were performed. rNCSCs and SCSCs lack genes expressed in fore/midbrain such as Emx1, Foxg1 and Nr2e1. Genes expressed at the trunk region and spinal cord were detected in rNCSCs and SCSCs (Weber et al., 2015).

(Figure 12). In addition, Unsupervised Principal Component Analysis (PCA) of the gene expression profiles demonstrate that NSCs from cortex and cerebellum, such as radial glia progenitors (RGPs) and granule neuron progenitors (GNPs), do not cluster together with rNCSCs and SCSCs (Supplementary Figure 2). They do not share common gene expression characteristics. On the other hand all three probe sets of rNCSCs and SCSCs cluster together and share common gene expression characteristics, demonstrating the homogeneity and reproducibility of rNCSCs cultures (Supplementary Figure 2). These results demonstrate a complete reprogramming of rNCSCs to cells with a SCSC trunk spinal cord identity.

4.1.5 The reprogramming is induced by FGF and can be blocked by BMP4

NCSCs, derived from E12.5 DRG, are reprogrammed to cells with CNS identity (rNCSCs) in a proliferation medium containing growth factors EGF and FGF. For CNS stem cells it has been shown, that EGF and FGF inhibit regional and cell type-specific differentiation and also increase the developmental potential (Hack et al., 2004; Gabay et al., 2003). EGF and FGF induce the expression of Olig2 in virtually all CNS stem cells maintained in NS cultures and are required for self-renewal (Hack et al., 2004). Thus, it was of interest to analyze the role of EGF and FGF in the reprogramming process of rNCSCs. Dissociated DRG cells were cultured in a proliferation medium containing either both EGF and FGF or only EGF or FGF. 3rd passage NS were analyzed after differentiation, using the standard differentiation medium (see Materials and Methods, section 3.2.1.2.6 and Table 2).

In the presence of EGF and FGF O4/Olig2-coexpressing oligodendrocyte-like (Figure 13A) and GFAP-positive but p75-negative astrocytes are produced from 3rd passage NS (Figure 13J) (Binder et al., 2011).

When the cells were only cultured with EGF, the generation of secondary NS was prevented (Data not shown) (Binder et al, 2011). Under the same conditions 3rd passage NS were generated in embryonic spinal cord cultures that display CNS characteristics,

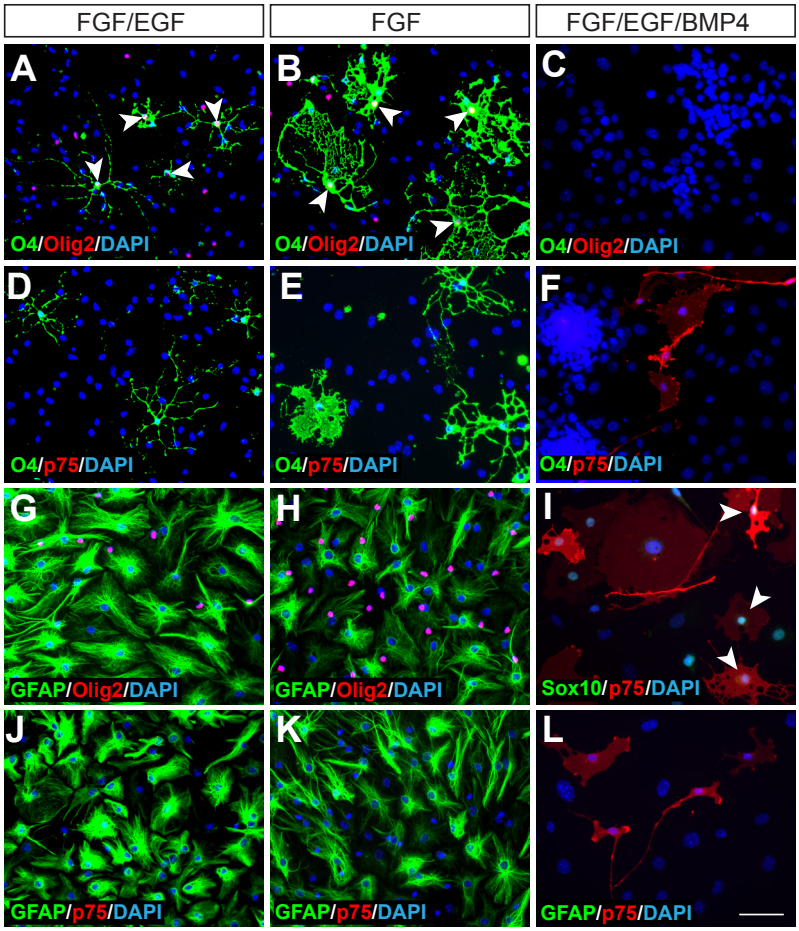

Figure 13: FGF is essential for the reprogramming and BMP4 blocks reprogramming of E12.5 DRG-derived NCSCs. 3rd passage rNCSCs cultured in the presence of EGF and FGF differentiated into O4/Olig2-positive oligodendrocyte-like cells (A) and GFAP-positive astrocyte-like cells (G and J) and are devoid of p75 (D and J). NCSCs cultured in the presence of FGF, differentiated into O4/Olig2-positive oligodendrocyte-like cells with immature morphology, (compare B,E and A,D), and are devoid of p75. They also differentiated into GFAP-positive astrocyte-like cells (H and K). When proliferation medium was supplement with BMP4, reprogramming towards CNS identity was prevented, demonstrated by the lack of Olig2-positive CNS cells (C). No O4- (C and F) and GFAP-positive glial (L) cells were observed under differentiation conditions. However, p75/ Sox10-positive neural crest derivatives were generated (I). Scale bar: 30 µm. (Left two columns were published in Binder et al., 2011 and right column in Weber et al., 2015).

such as O4 and Olig2 expression (Supplementary Figure 4).

FGF alone was sufficient for the generation of 3rd passage NS (FGF NCSCs) from DRG cultures. In addition, 3rd passage FGF NCSCs were able to differentiate into O4/Olig2-coexpressing oligodendrocyte-like cells (Figure 13B) albeit with less mature morphology in comparison to oligodendrocyte-like cells of EGF/FGF rNCSCs (compare Figure 13 A,D and B,E) (Binder et al., 2011). FGF NCSCs, like rNCSCs, generated GFAP-expressing astrocyte-like cells that lack p75 expression (Figure 13 H and K) (Binder et al., 2011). As FGF is essential for the growth of NCSCs in neurosphere cultures its function in the reprogramming to CNS fates remains unclear. Interestingly, embryonic spinal cord cell cultures generate 3rd passage NS in the presence of FGF

and generated oligodendrocyte progeny with mature morphology (Supplementary Figure 4).

In previous studies it has been shown that CEE (chicken embryo extract) in combination with BMP4 blocks the reprogramming of NCSCs towards a CNS identity. CEE is an established component of neural crest cultures (Molofsky et al., 2003; Calloni et al., 2009; Nagashimada et al., 2012). BMP4 plays a role in the early induction of the neural crest during development (reviewed in Meulemans and Bronner-Fraser, 2004) and elicits the transdifferentiation of CNS neural stem cells into neural crest cells (Gajavelli et al., 2004). In the present study it is demonstrated that BMP4 supplementation prevents Olig2 expression in 3rd passage NS (BMP NCSCs) (Figure 13C). Additionally, BMP NCSCs lack O4- (Figure 13C and F) and GFAP-positive glial cells (Figure 13L).They rather generate p75/Sox10-positive neural crest derivatives (Figure 13I (indicated with arrows)). Thus, the generation of rNCSCs is dependent on FGF but not EGF and is prevented by BMP4.

4.1.6 BMP4 represses the reprogramming of BMP NCSCs from passage 1 onwards

To understand the cellular mechanisms that prevent the reprogramming in the presence of BMP4, P1 (passage 1), P2 and P3 NS were analyzed in short-term cultures. The results indicate that the reprogramming is already efficiently antagonized by BMP4 at passage 1, as shown by the expression of p75 (Figure 14Aa,b,c and B) and Sox10 (Figure 14A,d,e,f and B) and the lack of the CNS marker gene Olig2 and O4 (Figure 14Ag,h,i). A high fraction of BMP NCSCs express Sox10 from passage 1 onwards (P1: 65.6% ± 6.0%, P2: 61.3% ± 3.5%, P3 45.5% ± 1.8% (n=3; mean ± SEM)) (Figure 14B), in contrast to rNCSCs which rapidly lose Sox10 expression in NS culture (Binder et al., 2011) (Figure 14B). p75 remains expressed only in a small subpopulation of BMP NCSCs (DRG starting population: 96.5% ± 0.5%, P1: 5.4% ± 1.3%, P2: 6.4% ± 2.8%, P3: 5.3% ± 2.3% (n=3; mean ± SEM)) (Figure 14B).

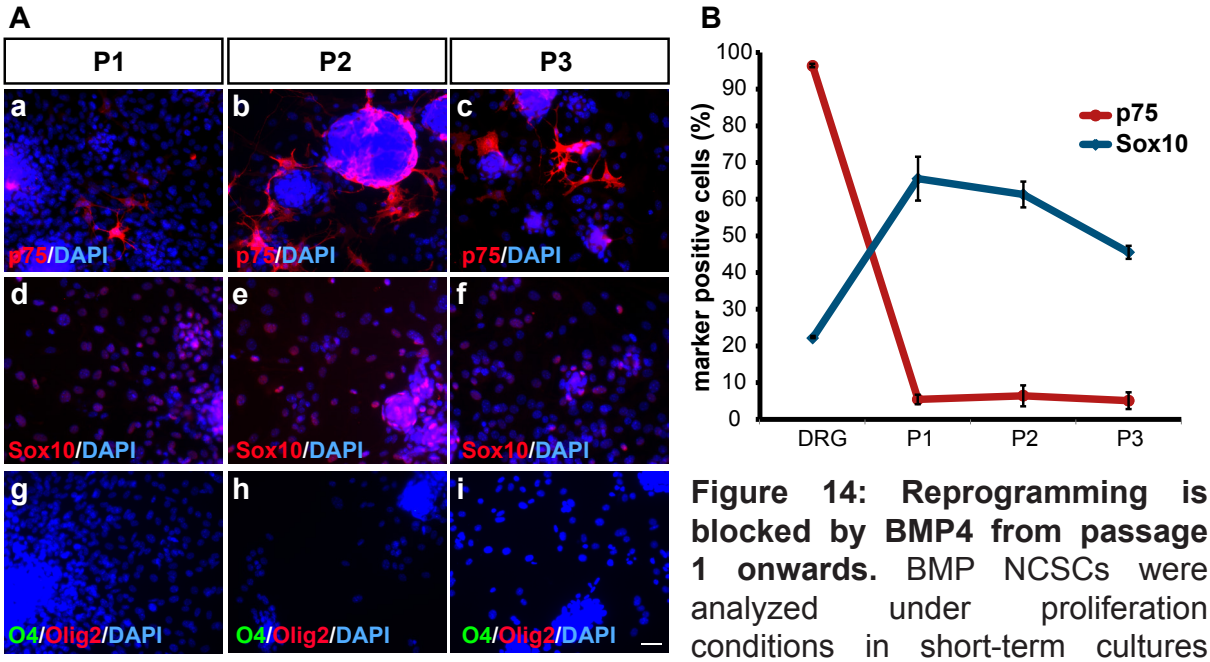

Figure 14: Reprogramming is blocked by BMP4 from passage 1 onwards. BMP NCSCs were analyzed under proliferation conditions in short-term cultures from passage 1 (P1) onwards via immunocytochemistry. A high fraction of BMP NCSCs express Sox10 (DRG starting population: 22.1% ± 1.7%, P1: 65.6% ± 6.0%, P2: 61.3% ± 3.5%, P3: 45.5% ± 1.8% (n=3; mean ± SEM)) (Ad,e,f and B). A small subpopulation of BMP NCSCs express p75 (DRG starting population: 96.5% ± 0.5%, P1: 5.4% ± 1.3%, P2: 6.4% ± 2.8%, P3: 5.3% ± 2.3% (n=3; mean ± SEM)) (Aa, b, c and B). BMP NCSCs are devoid of Olig2- and O4-positive cells at all passages (Ag,h,i). Scale bar: 30 µm. (Weber et al., 2015).

4.1.7 BMP NCSCs display strong differences in gene expression pattern in comparison to rNCSCs

The analysis of marker gene expression indicates that BMP NCSCs maintain their neural crest identity. For a more global comprehensive analysis, Affymetrix microarrays were carried out comparing the transcriptome of 3rd passage BMP NCSCs with 3rd passage rNCSCs. BMP NCSCs and rNCSCs display strong differences between their gene expression patterns, demonstrated via a scatter plot of the Affymetrix microarray data (Figure 15A). 28% of all probe sets are significantly differentially expressed (1.5 fold-change, adjusted p-value < 0.05). The PCA analysis demonstrates that BMP NCSCs do not cluster together with rNCSCs and SCSCs (Supplementary Figure 2). Whereas rNCSCs express genes at a high level that play a role in neural plate development, ventricular zone and neuroepithelial cells, such as Fabp7, Ptprz1, Tnc, Olig1, Olig2,

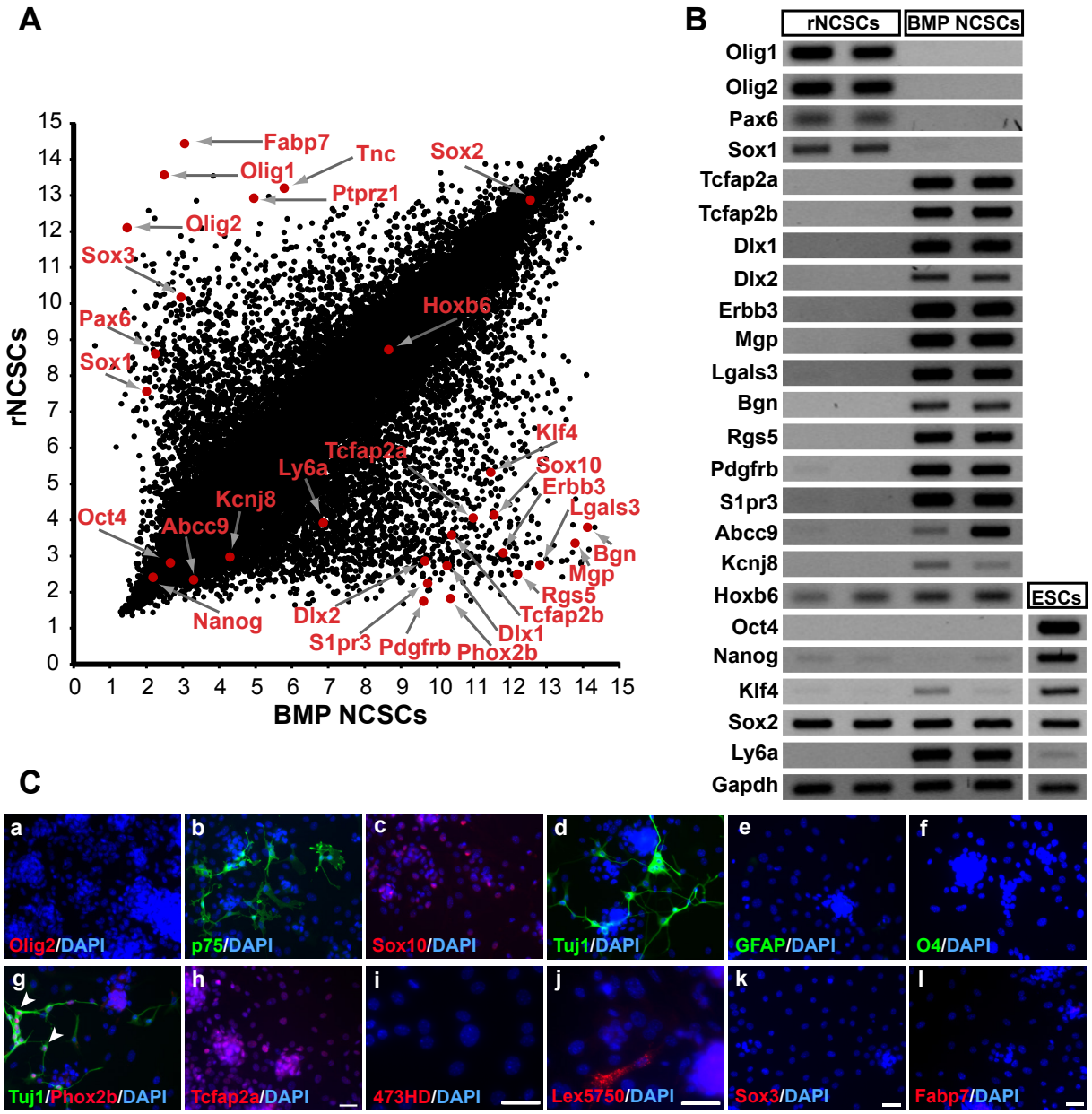

Figure 15: BMP NCSCs maintain the PNS identity and show strong differences in gene expression pattern compared to rNCSCs. (A) Scatter plot of microarry data sets demonstrates strong differences between gene expression profiles of BMP NCSCs and rNCSCs. CNS stem cell and neural crest marker genes are indicated (red). (B) Differential expression of marker genes is confirmed by RT-PCR (CNS marker: Olig1, Olig2, Pax6 and Sox1; Neural crest/neural crest-derivative marker: Tcfap2a/b, Dlx1/2, Erbb3, Mgp, Lgals3 and Bgn; MSC marker: Rgs5, Pdgfrb, S1pr3, Abcc9 and Kcnj8). Sox2 is strongly expressed in BMP NCSCs, rNCSCs and ESCs, whereas Oct4, Nanog and Klf4 are only expressed at high levels in ESCs. Trunk AP identity is shown by Hoxb6 expression. (C) Passage 3 BMP NCSCs generate p75-positive and Tuj1-positive cells with neuronal morphology (b,d) and Tuj1/Phox2b-positive autonomic neurons (g; arrows). O4- and GFAP-positive glial cells were not observed (e,f). BMP NCSCs are devoid of CNS markers Olig2 (a), 473HD (i), Sox3 (k) and Fabp7 (l). Lex5750 (j) is expressed in very few cells. BMP NCSCs express neural crest markers Tcfap2a (h) and Sox10 (c). Scale bar: 30 µm. (Weber et al., 2015).

Sox1, Sox2, and Sox3 (Figure 15a and Table 14), BMP NCSCs express those genes at background or undetectable levels. However, genes expressed in neural crest and neural crest derivatives, such as Sox10, Tcfap2a, Tcfap2b, Dlx1, Dlx2, Erbb3, Phox2b, Mgp, Lgals3 and Bgn (Figure 15a) were also expressed in BMP NCSCs at a high level. Interestingly, Gene Ontology (GO) analysis of the microarray data demonstrated an enrichment of genes characteristic for non-neural tissues, such as vasculature, cartilage and bone, in BMP NCSCs (Table 15). Analyzing the data in more detail, genes expressed in MSCs, perivascular pericytes and vascular smooth muscle cells, such as Pdgfrb, Rgs5, S1Pr3, Acta2, Spp1, Timp2, CD248 and Ly6a (Bondjers et al., 2003; Bondjers et al., 2006; Ishii et al., 2012; Lozito and Tuan, 2010; Kong et al., 2014; reviewed in Armulik et al., 2011), were strongly expressed in BMP NCSCs (Table 14 and Figure 15A and B). Furthermore, genes that contribute to chondrocyte and osteocyte development, including Bgn, Mgp, Lgals3, Col2a1, Col9a1, Fmod, and Acan (Suzuki et al., 2006; Zhang et al., 2003; Embree et al., 2010; reviewed in Emans and Peterson, 2014), were expressed at high levels in BMP NCSCs (Table 14 and Figure 15A and B). Of note, mesenchymal derivatives of the neural crest are restricted to the cranial neural crest in vivo but can be induced also from the trunk neural crest in vitro (John et al., 2011). In the context of mesenchymal differentiation it is of interest that the microarray data reveal high expression of BMPs in BMP NCSCs (Supplementary Figure 5). Interestingly, Id2 and Id4 are strongly expressed in BMP NCSCs, whereas in rNCSCs only Id2 is expressed at high levels (Supplementary Figure 5). Id genes belong to the inhibitor of differentiation (Id) family and their expression is controlled through the canonical pathway of BMP signaling (reviewed in Miyazono et al., 2005).
The expression of genes indicated (red) in the scatter plot was confirmed via RT-PCR (Figure 15B) and/or immunocytochemistry in short term cultures of 3rd passage rNCSCs (Figure 15C). The immunocytochemistry displayed high expression of the neural crest marker Tcfap2a in about 79% ± 4% BMP NCSCs (Figure 15Ch, Supplementary Figure 6). A subpopulation of 7% ± 3% (n=3) BMP NCSCs expresses the autonomic progenitor marker Phox2b (reviewed in Rohrer, 2011) and Tuj1/Phox2b-

Comparison of gene expression of rNCSCs and BMP NCSCs

Gene symbol	Gene name	Fold change	P-value
Early neural genes (neural plate)			
Zic1	zinc finger protein of the cerebellum 1	10.12	5.63E-10
Sox3	SRY-box containing gene 3	7.21	2.35E-08
Gbx2	gastrulation brain homeobox 2	4.73	0.00016
Sox11	SRY-box containing gene 11	7.18	3.38E-08
Pou3f1 (Oct6)	POU domain, class 3, transcr. factor 1	5.78	1.77E-05
Pou3f4 (Brn4)	POU domain, class 3, transcr. factor 4	5.65	1.08E-10
Spinal cord ventricular zone			
Fabp7	fatty acid binding protein 7, brain	11.38	3.14E-11
Olig1	oligodendrocyte transcription factor 1	11.07	7.31E-11
Olig2	oligodendrocyte transcription factor 2	10.62	2.97E-11
Pax6	paired box gene 6	6.36	1.54E-08
Sox1	SRY-box containing gene 1	5.56	1.24E-06
Slc1a3	Glial high affinity glut. transp. (Glast)	5.70	5.61E-05
Ptprz1	protein tyrosine phosphatase, receptor z	10.50	6.44E-11
Tnc	tenascin C	9.05	1.11E-08
Neural crest			
Snai2	snail homolog 2 (Drosophila)	-9.89	2.87E-10
Erbb3	v-erb-b2 homolog 3	-8.72	2.55E-10
Sox10	SRY-box containing gene 10	-7.42	8.50E-10
Tcfap2a	transcription factor AP-2, alpha	-6.93	7.04E-07
Tcfap2b	transcription factor AP-2, beta	-6.80	4.07E-05
Dlx2	distal-less homeobox 2	-6.79	1.21E-09
Chondrocyte/osteoblast			
Bgn	biglycan	-11.12	2.97E-11
Mgp	matrix gla protein	-10.43	3.03E-09
Lgals3	galectin	-10.07	2.66E-09
Col2a1	collagen, type II, alpha 1	-10.99	2.97E-11
Col9a1	collagen, type IX, alpha 1	-9.23	4.38E-08
Fmod	fibromodulin	-11.02	5.78E-10
Acan	aggrecan	-9.21	5.99E-08
Ogn	osteoglycin	-9.58	6.15E-08
MSC/pericyte			
Pdgfrb	Pdgf receptor, beta	-7.88	3.26E-08
Rgs5	Regulator of G-protein signaling 5	-9.71	4.54E-09
S1pr3	Endothelial diff. spingolipid G-pr. coupl. rec 3	-7.49	2.79E-09
Acta2	Smooth muscle actin, alpha 2	-5.9	9.81E-05
Spp1	Secreted phosphoprotein 1	-5.39	1.71E-06
Timp2	Tissue inhibitor of metalloprotease 2	-4.77	5.00E-06
CD248	Endosialin	-2.19	4.47E-05
Ly6a	Lymphocyte antigen 6 comp.; Sca-1	-2.9	0.0098

Table 14: Analysis of the gene expression in rNCSC and BMP NCSC neurospheres by Affymetrix microarrays. The following genes were selected for display: *i)* genes expressed in neural plate epithelial cells *ii)* in the spinal cord ventricular zone, *iii)* in neural crest *iv)* in mesenchymal neural crest derivatives, *v)* in MSC/perivascular cells. The average signal fold change is shown (log2-fold values). P-values were adjusted for multiple hypothesis testing (Benjamin and Hochberg, 1995) (Weber et al., 2015).

Gene ontology of genes differentially expressed in BMP NCSCs relative to rNCSCs

Cluster	GO term	Fold enrichment	P-value
Annotation cluster 1 (Enrichment score 17.27)			
GOTERM_BP-FAT	GO:0001944~vasculature development	3.06	4.96E-21
GOTERM_BP-FAT	GO:0001568~blood vessel development	3.02	6.64E-20
GOTERM_BP-FAT	GO:0048514~blood vessel morphogenesis	3.11	1.83E-17
GOTERM_BP_FAT	GO:0001525~angiogenesis	3.30	1.33E-13
Annotation cluster 2 (Enrichment score 10,03)			
GOTERM_BP_FAT	GO:0001501~skeletal system development	3.01	5.63E-23
GOTERM_BP_FAT	GO:0060348~bone development	2.85	1.16E-08
GOTERM_BP_FAT	GO:0001503~ossification	2.82	1.17E-07
GOTERM_BP_FAT	GO:0001649~osteoblast differentiation	2.78	9.84E-04
Annotation cluster 3 (Enrichment score 9,95)			
GOTERM_BP_FAT	GO:0048736~appendage development	3.22	1.46E-11
GOTERM_BP_FAT	GO:0060173~limb development	3.22	1.46E-11
GOTERM_BP_FAT	GO:0035108~limb morphogenesis	3.25	1.93E-11
GOTERM_BP_FAT	GO:0035107~appendage morphogenesis	3.25	1.93E-11
GOTERM_BP_FAT	GO:0035113~embr. append. morph.	3.27	6.18E-10
GOTERM_BP_FAT	GO:0030326~embr. limb morphogenesis	3.27	6.18E-10
GOTERM_BP_FAT	GO:0048598~embryonic morphogenesis	1.98	7.63E-09
Annotation cluster 4 (Enrichment score 9,65)			
GOTERM_BP_FAT	GO:0035295~tube development	2.58	2.75E-14
GOTERM_BP_FAT	GO:0048729~tissue morphogenesis	2.58	4.70E-13
GOTERM_BP_FAT	GO:0001763~morph. of a branch. struct.	3.21	4.61E-12
GOTERM_BP_FAT	GO:0035239~tube morphogenesis	2.73	5.62E-11
GOTERM_BP_FAT	GO:0048754~branch. Morph. of a tube	3.21	3.59E-09
GOTERM_BP_FAT	GO:0002009~morph. of an epithelium	2.43	2.95E-08
GOTERM_BP_FAT	GO:0060562~epithelial tube morph.	2.77	1.02E-07
GOTERM_BP_FAT	GO:0060429~epithelium development	2.03	1.64E-07
Anotation cluster 5 (Enrichment score 8,99)			
GOTERM_BP_FAT	GO:0001763~morph. of a branch. struct.	3.21	4.61E-12
GOTERM_BP_FAT	GO:0048732~gland development	2.37	1.07E-08
GOTERM_BP_FAT	GO:0048732~gland morphogenesis	3.22	2.04E08

Table 15: Gene ontology of genes differentially expressed in BMP NCSCs relative to rNCSCs. Genes with at least two-fold (q≤0.05) differential expression in BMP NCSCs vs rNCSCs were analyzed, using DAVID (http://david.abcc.ncifcrf.gov). The top five most highly enriched clusters of GO terms are presented (Weber et al., 2015).

coexpressing neurons were also detected (Figure 15Cg, Supplementary Figure 6). The PNS identity of Phox2b-expressing cells is unclear as Phox2b is also expressed in autonomic CNS neurons (Kang et al., 2007; Tiveron et al., 1996). However, the coexpression of Phox2b and Tcfap2a characterizes these cells as PNS autonomic neurons (Supplementary Figure 6). Tcfap2a is expressed in premigrating and migrating neural crest and neural crest derivatives (reviewed in Hilger-Everheim et al., 2000; Schorle et al., 1996; Zhang et al., 1996). BMP NCSCs lack the CNS stem cell marker of 473HD (Figure 15Ci) and reveal expression of Lex5750 (Figure 15Cj) in very few

cells. Sox3- (Figure 15Ck), Olig2- (Figure 15Ca) and Fabp7-positive CNS cells (Figure 15Cl) were never detected and are restricted to rNCSCs. GFAP- (Figure 15Ce) and O4-positive glial cells (Figure 15Cf) were not found under proliferation conditions. BMP NCSCs maintain, as rNCSCs, their trunk AP identity as shown by the expression of Hoxb6 (Figure 15A and B). Furthermore, a transient pluripotency state of BMP NCSCs was excluded since pluripotency genes such as Oct4, Nanog and KLf4 were only expressed at background or undetectable levels (Figure 15A and B). The expression of neural crest/neural crest-derivative and MSCs/pericyte progenitor marker genes and the lack of CNS marker in BMP NCSCs suggests a heterogeneous cell population that consists of MSC/pericyte progenitors and NCSCs with the potential to generate neural progeny.

4.1.8 BMP NCSCs differentiate to Peripherin-positive PNS neurons

In NS of BMP NCSCs a low number of Tuj1/p75-coexpressing neurons was observed. Tuj1 marks immature neurons as well as mature neurons. A subpopulation of Tuj1-positive neurons coexpresses Phox2b, a marker known to be expressed in proliferating neuroblasts and postmitotic neurons of the sympathetic ganglia (reviewed in Rohrer, 2011). To provide additional evidence for the potential of BMP NCSCs to produce PNS neurons, differentiation condition established for iNCSCs were used (Personal communication with Widera, Institute for Cell Biology, University of Bielefeld, Bielefeld, Germany). After plating, BMP NCSCs NS were treated with EGF and FGF and differentiation was induced by a complex medium containing neurotrophins, such as BDNF, GDNF, NT3 and NGF as well as cAMP and ascorbic acid (see Materials and Methods, section 3.2.1.2.6.and Table 2). To identify more mature PNS neurons, the cultures were analyzed for the expression of Peripherin and TH (Tyrosin hydroxylase). Peripherin is expressed in many but not all postmitotic neurons of the DRG and the sympathetic nervous system (Troy et al., 1990). TH is expressed by neuroblasts and neurons of the sympathetic ganglia (reviewed in Rohrer, 2011). The presence of

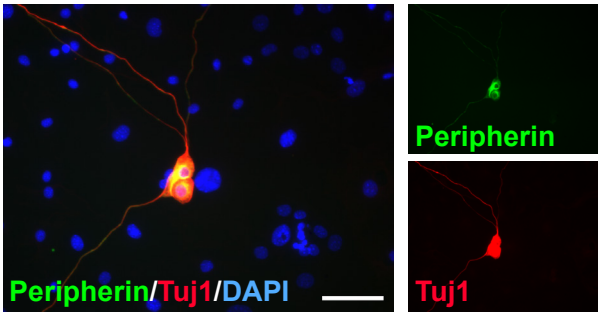

Figure 16: BMP NCSCs differentiate into Peripherin-expressing PNS neurons. Using a differentiation medium, containing neurotrophins for neuronal survival and differentiation, BMP NCSCs differentiate into Peripherin-expressing neurons. Scale bar: 30 µm. (Weber et al., 2015).

neurons coexpressing Tuj1 and Peripherin demonstrates the potential of BMP NCSCs to acquire a (more) mature PNS neuron phenotype (Figure 16). Notably, only a small number of Tuj1-positive neurons were generated and only a small number of them express both Tuj1 and Peripherin (Supplementary Figure 7). TH-expressing cells were not detected in BMP NCSCs (Data not shown).

4.1.9 BMP4 NCSCs in contrast to rNCSCs are able to differentiate into Schwann cell-like cells

The demonstration of PNS neurons in BMP NCSCs cultures raised the question if BMP NCSCs are able to acquire a PNS glia fate. O4-positive Schwann cells or satellite glia cells were not observed, neither under proliferation conditions in short term cultures nor under standard differentiation conditions (see Materials and Methods, Table 2). During the devolopment of the PNS, the survival and differentiation of Schwann cells are dependent on certain factors, secreted from neurons. Two of these factors, responsible for glial survival and maturation, are forskolin and neuregulin 1 (NRG1) (Wolpowitz et al., 2000; Morrissey et al., 1995; Yamada et al., 1995).Thus, a differentiation medium supplemented with NRG1 and forskolin was used to stimulate/elicit Schwann cell differentiation. 3rd passage BMP NCSCs treated with NRG1 and forskolin are indeed able to differentiate into O4/p75-positive PNS glial cells (Figure 17B). A PNS identity of these O4-positive glial cells is implicated not only by the coexpression of the PNS marker p75 but also by the lack of Olig2 expression (Figure 17F) and a Schwann cell morphology rather than oligodendrocyte morphology. These findings demonstrate

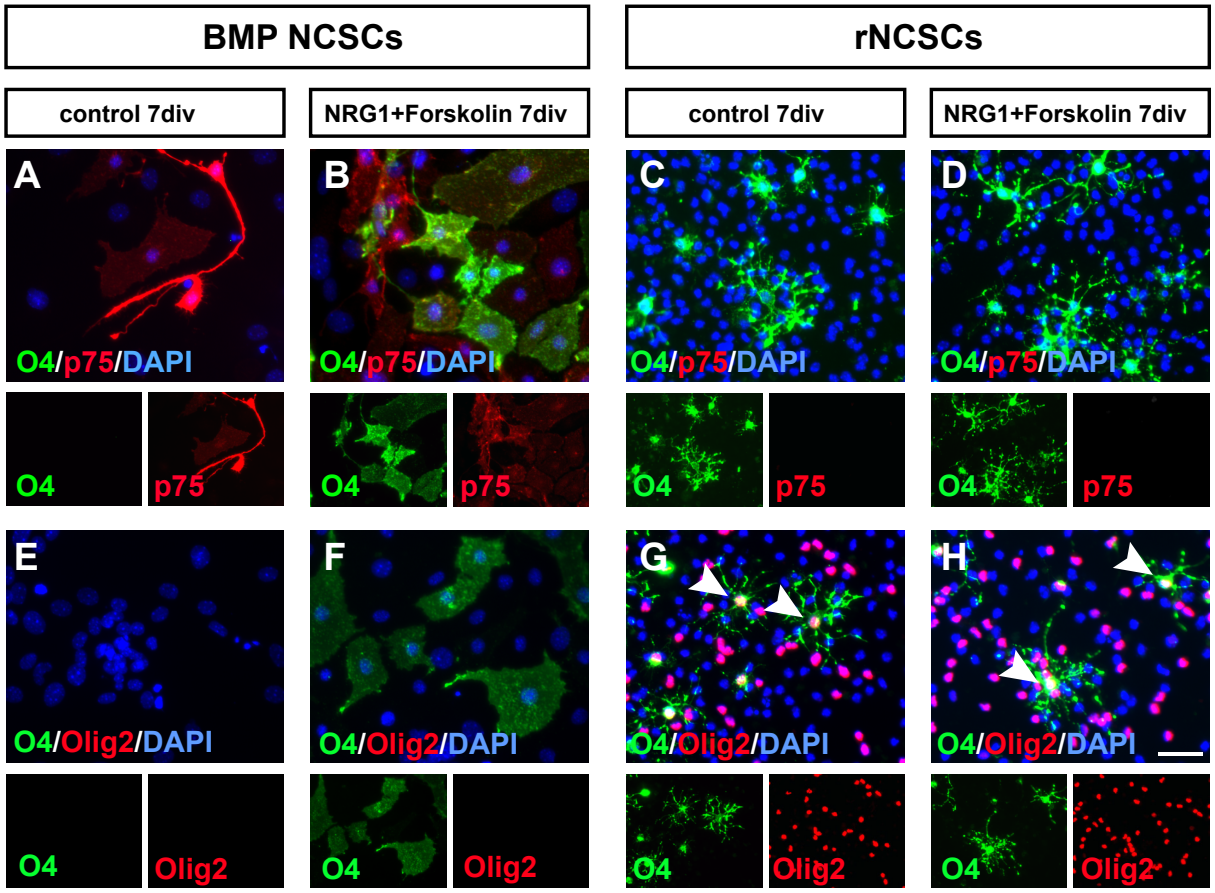

Figure 17: BMP NCSCs differentiate into O4/p75-coexpressing PNS glial cells. 3rd passage BMP NCSCs and rNCSCs were treated with NRG1 and forskolin for 7 days. In the presence of NRG1 and forskolin, BMP4 NCSCs differentiate into O4/p75-coexpressing PNS glial cells (B) that are devoid of Olig2 expression (F). Without NRG1 and forskolin treatment, BMP NCSCs do not differentiate into O4/p75-positive PNS glial cells (A) and lack Olig2-positive cells (E). rNCSCs cultures, treated with NRG1 and forsklin, are devoid of O4/p75-positive PNS glial cells and rather contain O4/Olig2-positive oligodendrocyte-like cells that lack p75 expression (D and H) like in the control (C and G). Scale bar: 30 µm. (B is published in Weber et al., 2015).

a neural crest-specific response of BMP NCSCs to NRG1 and forskolin i.e. to the generation and maintenance of Schwann cells.

In contrast, rNCSCs were not able to differentiate into O4/p75-coexpressing PNS glial cells (Figure 17D) upon treatment with NRG1 and forskolin. O4/Olig2-coexpressing and p75-negative oligodendrocyte-like cells with oligodendrocyte morphology were detected (Figure 17D and H). rNCSCs do not respond to Schwann cell survival and maturing factors such as NRG1 and forskolin, confirming a stable CNS identity of rNCSCs.

4.1.10 Delayed BMP4 removal from BMP NCSCs does not induce CNS marker gene expression in BMP NCSCs

The repression of the CNS reprogramming by BMP4 raises the question if BMP4 is continuously required for the maintenance of the neural crest identity in BMP NCSC cultures at later passages. In order to shed light on this question, BMP4 was removed from the proliferation medium. 3rd passage BMP NCSCs were dissociated into single cells and cultured in the absence of BMP4, using the same EGF/FGF proliferation medium as for rNCSCs (see Materials and Methods, Table2). Notably, in the absence

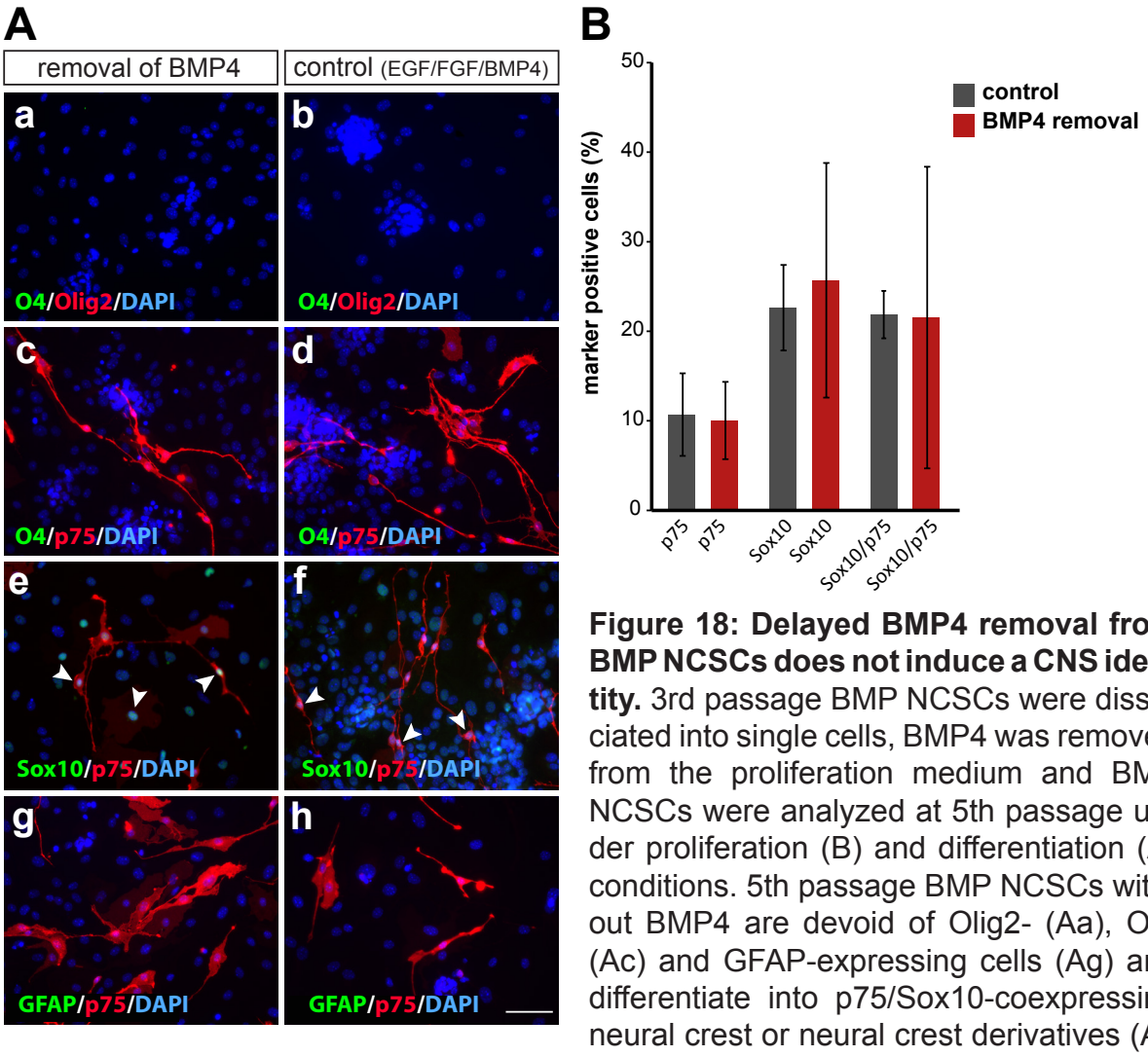

Figure 18: Delayed BMP4 removal from BMP NCSCs does not induce a CNS identity. 3rd passage BMP NCSCs were dissociated into single cells, BMP4 was removed from the proliferation medium and BMP NCSCs were analyzed at 5th passage under proliferation (B) and differentiation (A) conditions. 5th passage BMP NCSCs without BMP4 are devoid of Olig2- (Aa), O4- (Ac) and GFAP-expressing cells (Ag) and differentiate into p75/Sox10-coexpressing neural crest or neural crest derivatives (Ae and B), comparable to control BMP4 treated BMP NCSCs at the 5th passage (Ab, d, f, h and B). The proportion of p75- and Sox10-positive cells and p75/Sox10-double positive cells did not differ in BMP NCSCs with or without BMP4 (B). Scale bar: 30 µm. n=3; mean ± SEM. (Weber et al., 2015).

of BMP4, EGF and FGF induce CNS fates in rNCSCs. The cells were analyzed at the 5th passage under proliferation conditions in short-term cultures or under standard differentiation conditions (see Materials and Methods, Table 2). Of note, the proportion of p75-, Sox10-expressing as well as Sox10/p75-coexpressing cells was not changed upon BMP4 removal (Figure 18B). Interestingly, the proportion of Sox10-positive cells in 5th passage BMP NCSCs decreased (Figure 18B), compared to 3rd passage BMP NCSCs (see section 4.1.6). BMP NCSCs, maintained in the absence of BMP4, lack the CNS marker gene Olig2 (Supplementary Figure 8). Also under differentiation conditions, BMP NCSCs cultured without BMP4 did not generate Olig2-positive CNS cells (Figure 18Aa) nor O4- (Figure 18Aa and c) and GFAP-expressing glial cells (Figure 18Ae). Additionally, p75/Sox10-positive cells (Figure 18Ae, see arrows) were detected after differentiation. BMP NCSCs kept with or without BMP4 from passage 3 to passage 5 showed identical properties which suggests that the PNS identity of BMP NCSCs stays stable after the delayed BMP4 removal.

4.1.11 The CNS identity of rNCSCs stays stable after delayed BMP4 application

As BMP4 maintains the PNS identity of BMP NCSCs the question is raised if BMP4 is able to induce PNS traits in rNCSCs. Therefore, 3rd passage rNCSCs were dissociated into single cells and cultured as NS in a medium supplemented with BMP4 (BMP4/EGF/FGF). The effect of BMP4 addition was analyzed at the 5th passage in short term cultures. For the differentiation the standard differentiation medium was used without BMP4 (see Materials and Methods, Table 2). Interestingly, Olig2 is slightly down regulated in rNCSCs NS treated with BMP4, in comparison to the control rNCSCs without BMP4. However, Olig2 is still expressed in approximately 70% of the cells (Figure 19B). Furthermore, the CNS marker gene Sox3 is massively down regulated upon the BMP4 treatment of rNCSCs NS (Figure 19C). rNCSCs supplemented with BMP4 differentiated into GFAP-positive astrocyte-like cells that lack p75 expression (Figure 19Ae and g) like control rNCSCs without BMP4 (Figure 19Af and h). Additionally,

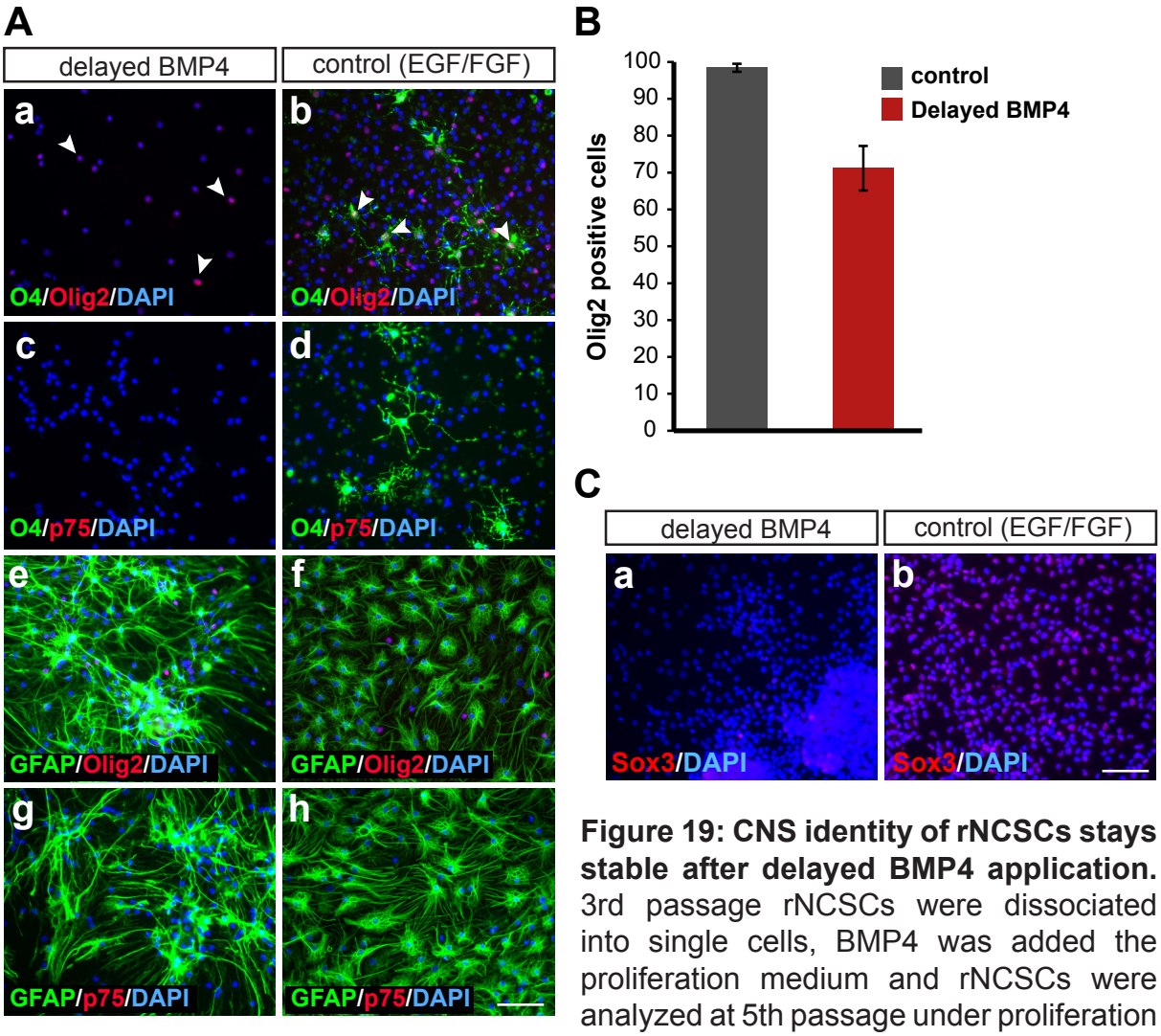

Figure 19: CNS identity of rNCSCs stays stable after delayed BMP4 application. 3rd passage rNCSCs were dissociated into single cells, BMP4 was added the proliferation medium and rNCSCs were analyzed at 5th passage under proliferation in short-term cultures (B and C) or after differentiation for 7 days (A). rNCSCs with BMP4 still differentiate into GFAP-positive astrocyte-like cells (Ae and g), express Olig2 (Aa) and lack p75-positive cells (Ac) like control rNCSCs without BMP4 (Ab, d, f and h). rNCSCs with BMP4 lack O4-positive oligodendrocyte-like cells (Aa and c). However, control rNCSCs generate O4/Olig2-expressing oligodendrocyte-like cells (Ab and d). Under proliferation conditions Olig2 is decreased in rNCSCs with BMP4 (B), compared to the control (B) but still expressed in many cells (n=3, mean ± SEM). (C) Sox3 is strongly downregulated in BMP4-treated rNCSCs. Scale bar: 30 µm. (Weber et al., 2015).

Olig2 was still expressed after differentiation (Figure 19Aa and e). In contrast to control rNCSCs without BMP4 (Figure 19Ab), rNCSCs with BMP4 lose the ability to differentiate into O4/Olig2-coexpressing oligodendrocyte-like cells (Figure 19Aa). The lack of oligodendrocyte-like cells could be caused by an effect of BMP4 on oligodendrocyte differentiation, which has previously been observed in the CNS (Samanta et al., 2004; Mekki-Dauriac, 2002; Miller et al., 2004). The results support the notion that the CNS

identity of rNCSCs, demonstrated by the expression of the CNS marker gene Olig2, the generation of astrocyte-like cells and the lack of p75, stays stable after delayed BMP4 application.

4.1.12 Is forced Olig2 expression able to induce oligodendrocyte generation in BMP NCSCs?

It has been described that the combined action of three transcription factors, Olig2, Sox10 and Nkx6.2 or Zfp536, is sufficient to induce oligodendrocyte marker genes in cultures of mouse and rat fibroblasts. Induced oligodendrocyte-like cells display a oligodendrocyte specific-morphology and are able to myelinate axons in *shiverer* or hypomyelinated mice. The combination of Olig2 and Sox10 also induced oligodendrocytes, although with lower efficiency (Najm et al., 2013; Yang et al., 2013). Passage 1 BMP NCSCs express Sox10 at a high level (approximately 65% of the cells express Sox10 (see section 4.1.6)), which raises the question whether Sox10 in combination with forced Olig2 expression is able to induce oligodendrogenesis. For forced Olig2 expression in BMP NCSCs, a *Piggy Bac* transposon plasmid containing *mmOlig2* (pPB-CAG.Olig2-puDtk) was used. *Piggy Bac* transposons integrate into the host genome but can be removed by the transfection of cells with a transposase-expressing vector. The transient transgene introduction the via *Piggy Bac* transposon is expected to avoid permanent genetic alteration, in contrast to the permanent retroviral introduced genes, for example the retrovirally introduced pluripotency genes in iPSCs (reviewed in Ben-David and Benvenisty, 2011).

Primary BMP NCSCs were dissociated into single cells and were transfected with the *Piggy Bac* transposon containing *mmOlig2* (see Materials and Methods see section 3.2.1.2.7). In order to check the efficiency of the transfection, the cells were plated on poly-DL-ornithine laminin coated 4-well plates in a medium, containing EGF and FGF, and stained for Olig2 16 h after transfection. The transfection rate determined from the number of Olig2-positive cells was about 15% ± 0,1% (Figure 20A and B).

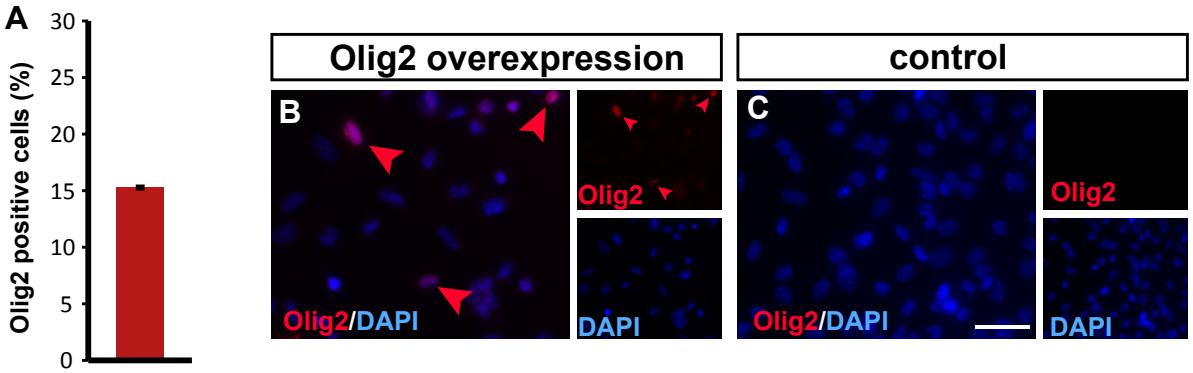

Figure 20: BMP NCSCs express Olig2 after Olig2 transfection. Primary BMP NCSCs were dissociated into single cells and transfected with *Piggy Bac* transposon plasmid containing *mmOlig2*. Cells were cultured with EGF/FGF on poly-DL-ornithine laminin coated 4-well pates and analyzed 16 h after transfection. The transfection efficiency was about 15% ± 0,1% (n=2) (A and B). Control, untransfected cells did not express Olig2 (C). Mean ± SEM. Scale bar: 30 µm. Red arrows indicate Olig2-positive cells.

The Olig2 transfected cells and control BMP NCSCs were cultured for 11 days in the presence of EGF and FGF for the generation of NS and were either analyzed in short term cultures or after differentiation in the presence of a oligodendrocyte differentiation medium. Although virtually all cells express Sox10 in these cultures, oligodendrocyte-like cells were not observed in NS short term cultures and after differentiation (Data not shown), according to Njam et al., (2003) and Yang et al., (2013). The high proportion of Sox10-positive cells in BMP NCSC cultures maintained without BMP4 after passage1 suggests that the Sox10 expression is reduced by BMP4 between passage 1 and passage 3 (Data not shown). Using substrate attached rather than floating NS cultures and a medium conditioned by rNCSCs a low number of oligodendrocyte-like cells was detected in Olig2-transfected cultures. After 18 days in a proliferation medium and after 7 days of standard differentiation without RA (see Materials and Methods, section 3.2.1.2.6 and Table 2) O4/Olig2-coexpressing cells with oligodendrocyte specific-morphology were detected in the Olig2-transfected cell fraction (Figure 21A and B (O4/Olig2-coexpressing cells are indicated with white arrows)). As a control, *Piggy Bac* transposon plasmid, containing *mmNkx6.2*-transfected cells were used. Although, Nkx6.2 is able to induce Olig2 expression in the pMN domain of the ventral spinal cord (Bennett et al., 2001; Lui et al., 2003; Novitch et al., 2001; Sun et al., 1998) neither Olig2 nor O4 was induced by Nkx6.2 in BMP NCSCs (Figure 21C). This

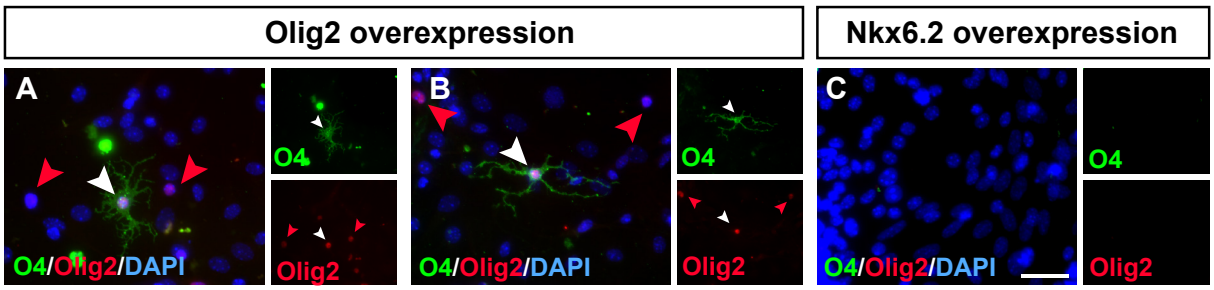

Figure 21: Forced expression of Olig2 induces O4/Olig2 coexpression in BMP NCSCs with oligodendrocyte morphology. Freshly Olig2 transfected cells were cultured on poly-DL-ornithine laminin coated 4-well plates for 18 days under proliferation conditions and were analyzed after differentiation for 7 days. Olig2 transfected BMP NCSCs generated very few O4/Olig2-coexpressing cells with oligodendrocyte morphology (A and B, white arrows). As a control Nkx6.2 transfected cells were used, that did not induce Olig2 and O4 expression (C). White arrowheads indicate double-positive cells and red arrows single Olig2-positive cells. Scale bars: 30 µm. n=1.

preliminary result represents the proof of the principle that oligodendrocyte development may be induced by Olig2 expression in BMP NCSCs. However, these results must be confirmed by using more markers.

4.2 NCSCs derived from postnatal DRG are able to generate CNS cell types in vitro

NCSCs derived from E12.5 DRG can be reprogrammed to cells with CNS identity in a medium containing EGF and FGF. To investigate whether NCSCs are maintained during development and keep the potential for CNS reprogramming, NCSCs from postnatal (P3) DRG were obtained. It will be analyzed if NCSCs from postnatal (P3) DRG acquire CNS fates, as observed for embryonic rNCSCs.

4.2.1 NCSCs from postnatal (P3) DRG acquire the CNS marker Olig2 but the PNS marker p75 is maintained in NS culture under proliferation conditions

The properties of 3rd passage NS cells from postnatal mouse DRG were analyzed in short-term cultures (see Materials and Methods, section 3.2.1.2.5). Virtually all cells express Olig2 (Figure 22A). p75 was massively reduced but detectable in a

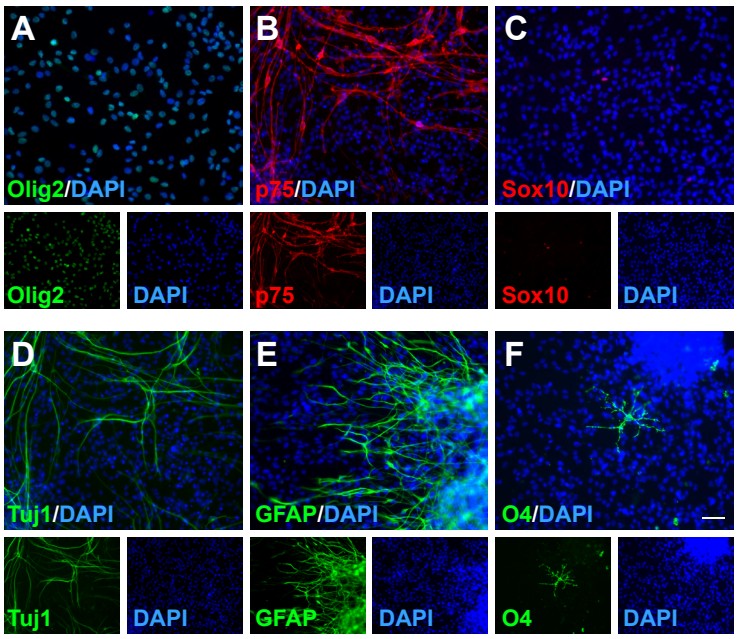

Figure 22: NCSCs from postnatal DRG acquire the CNS marker gene Olig2, however, the PNS marker p75 is maintained. 3rd passage NS from P3 DRG were analyzed under proliferation conditions in short-term cultures. Virtually all NS cells express Olig2 (A). p75 (B) and Sox10 expression was detected in a low number of cells (C). Tuj1-positive neurons (D), GFAP-positive astrocytes (E) and a few O4-positive oligodendrocyte-like cells (F) were generated.
Scale bars: 30 µm (n=2).

low number of cells (Figure 22B), in contrast to rNCSCs which completely lack p75 expression at passage 3. Additionally, Sox10-expressing cells were detected (Figure 22C). Differentiated cell types, such as Tuj1-positive neurons (Figure 22D) and GFAP-positive glial cells that display an astrocyte morphology (Figure 22E) were found in low numbers. O4-positive glial cells with oligodendrocyte morphology emerged (Figure 22F), in contrast to rNCSCs that lack O4 expression under proliferation conditions (see section 4.1.2). This leads to the conclusion that progenitor cells from postnatal DRG can be propagated in medium containing EGF and FGF which results in the acquisition of Olig2 and the loss of PNS markers in the vast majority of cells.

4.2.2 3rd passage NS derived from postnatal (P3) DRG differentiate into CNS cell types including oligodendrocyte and astrocyte progeny

To analyze the differentiation potential of postnatal derived NCSCs, 3rd passage NS were treated with the standard differentiation medium (see Materials and Methods, section 3.2.1.2.6 and Table 2). As observed for embryonic DRG-derived NS GFAP-expressing glial cells with astrocyte morphology, that lack the neural crest marker

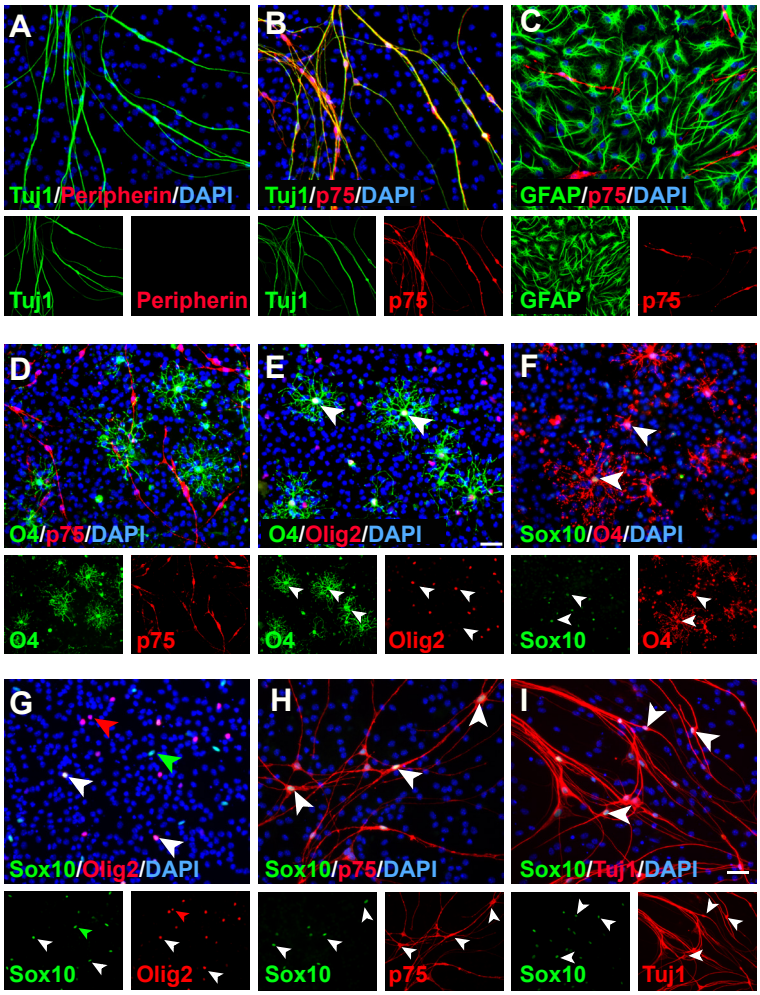

Figure 23: 3rd passage NS cells derived from P3 DRG differentiate to CNS cell types and PNS neurons. 3rd passage NS differentiate to Tuj1-positive neurons that are devoid of Peripherin (A) but coexpress p75 (B). GFAP-positive astrocyte-like cells (C) and O4-positive oligodendrocyte- like cells (D) are devoid of p75 expression. O4-expressing oligodendrocyte-like cells coexpress Olig2 (E) and some O4- positive cells co-express Sox10 (F). Sox10/ Olig2-coexpressing cells were detected (indicated with white arrowheads) as well as only Olig2- (indicated with red arrowheads) and Sox10- (indicated with green arrowheads) expressing cells (G). p75- (H) and Tuj1-positive cells (I) co-express Sox10. Scale bars: 30 µm. n=5 (A - E published in Binder et al., 2011).

p75, could be identified (Figure 23C). The emergence of oligodendrocyte-like cells was indicated by the coexpression of O4 and Olig2, the morphology characteristic for oligodendrocytes (Figure 23E) as well as the lack of p75 expression (Figure 23D). O4-positive cells that coexpress Sox10 were also detected (Figure 23F). Some Sox10-positive cells coexpress Olig2, which suggests the generation of OPCs (Figure 23G (indicated with white arrowheads)). However, also marker combinations typical for neural crest and neural crest progeny were detected as cells that coexpress Sox10 and p75 or Tuj1 and Sox10 (Figure 23H and I). Although the Tuj1/p75-coexpressing neurons lack Peripherin expression (Figure 23A) they are tentatively characterized as immature PNS neurons by the coexpression of Tuj1 and Sox10 (Figure 23I). In the stellate ganglion Sox10 is coexpressed with Tuj1-positive proliferating neuroblasts (Gonsalvez et al., 2012).

These findings confirm that progenitors from postnatal-derived DRG differentiate into CNS cell types, including oligodendrocyte-like cells and astrocyte-like cells, but maintain the potential to generate PNS cells such as immature PNS neurons. This implies that postnatal DRG derived NCSCs display a more restricted developmental plasticity when compared with NCSCs from embryonic DRG.

4.3 Do adult NCSCs from mouse palate acquire CNS fates?

The use of rNCSCs for cell therapies requires an accessible source of these cells in the adult organism. As the DRG from adult mouse is not an easily accessible tissue, it was of interest to identify an alternative source. An easily accessible tissue source that contains NCSCs is the adult rat palatal rugae. Widera et al., (2009 and 2011) identified Nestin/p75/S100-positive neural crest-derived cells adjacent to Meissner corpuscles within the palate of adult Wistar rats. These so-called palatal neural crest-related stem cells (pNCSCs) can be propagated in NS culture for at least 20 passages. Primary NS revealed a high expression of Sox2, Nestin, p75, Slug, Twist and Sox9. A few cells expressed GFAP and pluripotency genes, such as Oct4, Klf4 and c-Myc, were also detected. pNCSCs differentiated into neurons and glial cells in vitro (Widera et al., 2009; Widera et al., 2011). However, it was unclear if NCSCs, derived from adult mouse palate (pNCSCs), acquire CNS fates in NS culture. In addition a direct proof for neural crest origin of pNCSCs was lacking.

4.3.1 Progenitor cells from adult mouse palate (pNCSCs) are derived from Sox10-positive NCSCs or glial cell

To investigate whether neurosphere forming cells in dissociated adult mouse palate are derived from the neural crest, the palate was dissociated from a *Sox10-GFP* mice (see Materials and Methods, section 3.2.1.1.4). In this mouse strain, GFP is expressed under the control of the Sox10 promotor which allows to identify Sox10-expressing neural

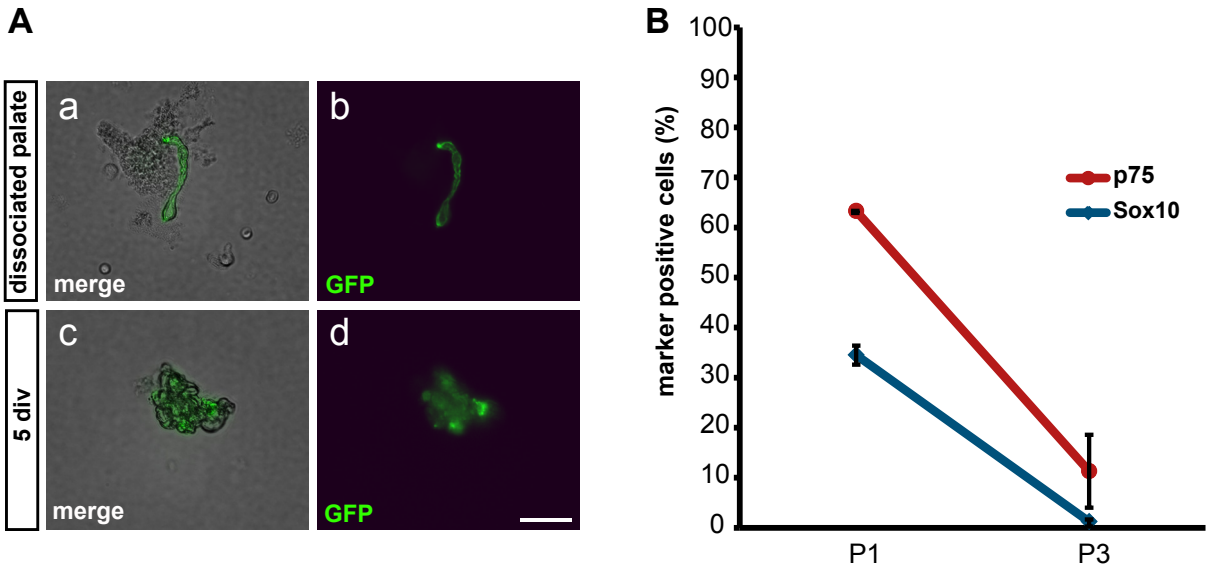

Figure 24: NS forming cells were derived from Sox10-positive NCSCs or Schwann cells and Sox10 was downregulated during NS culture. GFP-Sox10-positive freshly isolated palatal cells display Schwann cell morphology (Aa and b). After 5 days in culture the majority of NS were GFP-positive (Ac and d). p75 (P1: 63.3% ± 0.2%, P3: 11.3% ± 7.3%, n=3; mean ± SEM)) and Sox10 (P1: 34% ± 1.9%, P3: 1.2% ± 0.7% (n=3 to 5; mean ± SEM)) are downregulated during NS culture (B). Scale bars: 30 µm. (Weber et al., 2015).

crest cells and Schwann cells. Sox10-GFP-positive cells from freshly isolated palate displayed a Schwann cell morphology (Figure 24Aa and b). The cells were cultured in a proliferation medium (see Materials and Methods, section 3.2.1.2.4 and Table 2). After 5 days in culture, small aggregates, displaying a NS morphology, appeared and 94.5% ± 2% of NS displayed GFP expression (Figure 24Ac, d). The proportion of NS-containing GFP-positive cells strongly decreased after 7 days in culture, when only 52% ± 2% of the NS contained GFP-positive cells (Data not shown). Furthermore, the proportion of Sox10-positive cells was reduced during NS culture from 34% ± 1.9% in passage 1 NS cells to 1.2% ± 0.7% in passage 3 (Figure 24B), analyzed in short term cultures. The neural crest/neural crest-derivative marker p75 was expressed at high levels in passage 1 NS, when about 63.3% ± 0.2% of the cells express p75 and subsequently p75 expression decreases so that only 11.3% ± 7.3% of the cells express p75 in passage 3 NS (Figure 24B). This result supports the assumption that palate-derived NS are generated from Sox10/p75-positive NCSCs or Schwann cells

that downregulate these neural crest markers p75 and Sox10 during NS culture.

4.3.2 3rd passage pNCSCs lack the expression of the CNS marker gene Olig2

3rd passage pNCSCs NS cells were analyzed for the expression of CNS versus PNS marker genes. Interestingly, pNCSCs lack the CNS marker Olig2 (Figure 25a) and no O4-(Figure 25f) and GFAP-expressing CNS or PNS glial cells (Figure 25e) were found. A low number of p75- (Figure 25b) and Sox10-positive neural crest derivatives (Figure 25c) and Tuj1/p75-coexpressing PNS neurons (Figure 25d) were detected. A similar result was observed after the differentiation of pNCSCs i.e. the lack of Olig2 (Figure 25g), the absence of O4-(Figure 25l) and GFAP-positive glial cells (Figure 25k), the generation of a few cells expressing p75 (Figure 25h) and Sox10 (Figure 25i) as well as Tuj1/p75-coexpressing neurons (Figure 25j). Due to the fact that the majority of

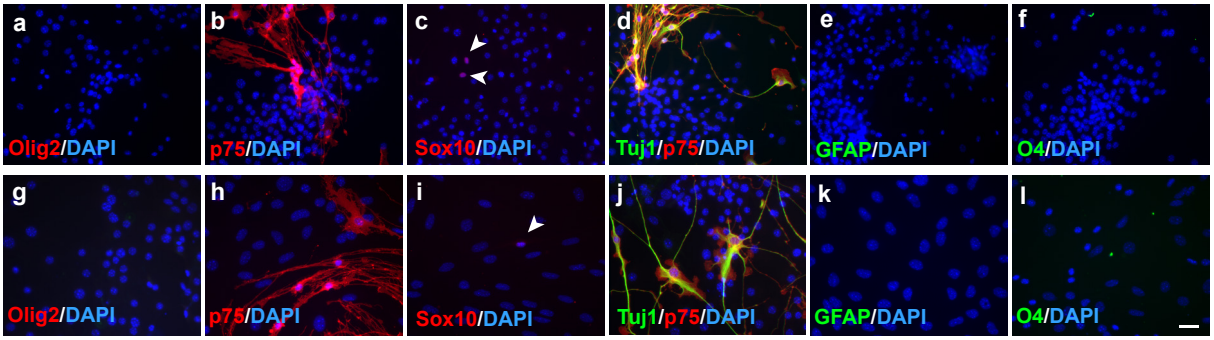

Figure 25: 3rd passage pNCSCs are devoid of the CNS marker gene Olig2. 3rd passage pNCSCs were analyzed under proliferation conditions in short-term cultures and under differentiation conditions via immunocytochemistry. Under proliferation conditions pNCSCs lack Olig2, GFAP and O4 expression (a, e, f). A few p75- (b), Sox10- expressing (c) and Tuj1/p75-coexpressing cells (d) were detected. Also after differentiation pNCSCs lack Olig2 expression (g) and GFAP- and O4-positive glial cells are absent (k,l). Only few p75- (h) and Sox10-positive cells (i) as well as Tuj/p75-coexpressing cells (j) were detected. Scale bars: 30 µm. n=3. (Weber et al., 2015).

cells were negative for marker genes such as Olig2, p75, Sox10, GFAP and O4, the cell type identity of most pNCSCs was unclear.

4.3.3 pNCSCs acquire cranial neural crest fates

In order to identify pNCSCs the gene expression profile was analyzed by an Affymetrix microarray, of pNCSCs and rNCSCs. The comparison revealed strong differences in the gene expression pattern as 31% of the probe sets are differentially expressed (1.5 fold change, adjusted p-value <0.05) (Figure 26A). PCA analysis demonstrate that pNCSCs do not cluster together with rNCSCs and SCSCs (Supplementary Figure 2). pNCSCs are devoid of the CNS or neural plate marker genes (Fabp7, Olig1, Olig2, Pax6, Sox1) that are selectively expressed by rNCSCs (Figure 26A). However, pNCSCs express genes at a high level which are expressed in neural crest/neural crest-derivatives such as Tfap2a, Dlx1, Dlx2 and Erbb3 (Figure 26A). The GO analysis revealed for pNCSCs an enrichment of the GO terms vasculatory development, blood vessel development and morphogenesis and angiogenesis (Table 17). Furthermore, pNCSCs express genes at a high level that are found in MSCs/pericyte progenitors, such as Pdgfrb, Rgs5, Abcc9, S1pr3, Kcnj8 and Ly6a (Figure 26A and Table 16). In the head region, the cranial neural crest generates, besides PNS progeny, mesenchymal derivatives such as cells in the meninges, brain, retina, optic nerve and thymus (reviewed in Le Douarin, 2004). Pericytes in the face and neck region are also neural crest derived (reviewed in Le Douarin, 2004). Interestingly, the vast majority of palatal mesenchyme is derived from the cranial neural crest (Ito et al., 2003). The expression of MSC and pericyte marker genes thus reflects the normal development of cranial neural crest stem cells and the generation of a subset i.e. mesenchymal derivatives. Furthermore, pNCSCs lack Hoxb6 expression, which is a trunk restricted marker gene (Figure 26A). Pluripotency genes are only weakly expressed except of Sox2 and Klf4 (Figure 26A). The differential expression of a number of genes, indicated in the scatter plot (red) was confirmed via RT-PCR (Figure 26B). These results lead to the assumption that

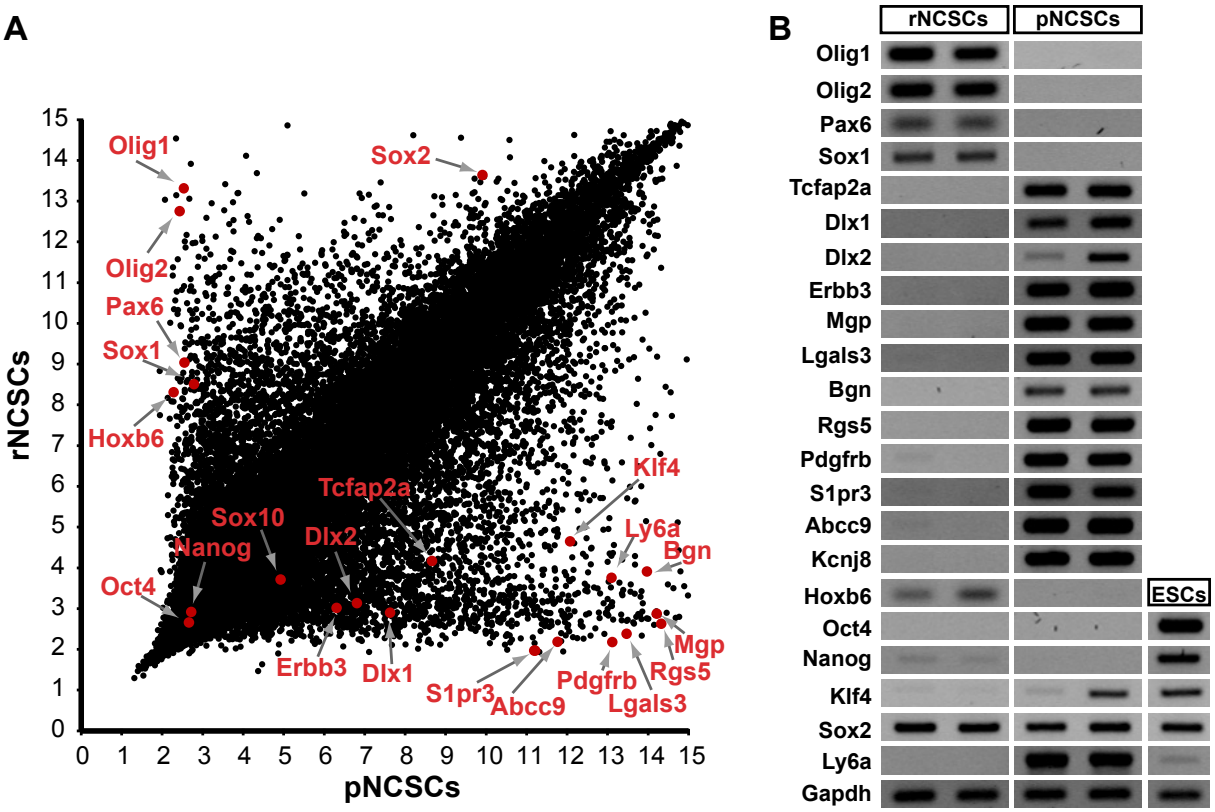

Figure 26: Gene expression profiling of pNCSCs and rNCSCs reveal strong differences in gene expression pattern. The scatter plot demonstrates strong differences in gene expression pattern of pNCSCs and rNCSCs (A). CNS stem cell and neural crest marker genes are indicated (red). (B) The differential expression of marker genes is confirmed by RT-PCR (CNS marker: Olig1, Olig2, Pax6 and Sox1; Neural crest/neural crest derivative marker Tcfap2a, Dlx1/2, Erbb3, Mgp, Lgals3 and Bgn); MSC marker: Rgs5, Pdgfrb, S1pr3, Abcc9 and Kcnj8). Sox2 is strongly expressed in pNCSCs, rNCSCs and ESCs. Klf4 is expressed in pNCSCs and ESCs. Nanog and Oct4 are only expressed at high levels in ESCs. Hoxb6 is only expressed in rNCSCs and is missing in pNCSCs. (Weber et al., 2015).

pNCSCs are not able to generate any CNS cell types, at least under the medium conditions used and are restricted to the generation of a subset of cranial neural crest derivatives.

Comparison of gene expression of rNCSCs and pNCSCs

Gene symbol	Gene name	Fold change	P-value
Early neural genes (neural plate)			
Zic1	zinc finger protein of the cerebellum 1	10.95	1.43E-09
Sox3	SRY-box containing gene 3	6.59	1.73E-08
Gbx2	gastrulation brain homeobox 2	4.65	0.00045
Sox11	SRY-box containing gene 11	9.15	1,61E-08
Pou3f1 (Oct6)	POU domain, class 3, transcr. factor 1	4.11	0.00011
Pou3f4 (Brn4)	POU domain, class 3, transcr. factor 4	5.12	1.82E-08
Spinal cord ventricular zone			
Fabp7	fatty acid binding protein 7, brain	9.75	7.46E-08
Olig1	oligodendrocyte transcription factor 1	10.78	1.31E-11
Olig2	oligodendrocyte transcription factor 2	10.32	3.37E-11
Pax6	paired box gene 6	6.48	4.25E-09
Sox1	SRY-box containing gene 1	5.72	7.99E-07
Slc1a3	Glial high affinity glut. transp. (Glast)	6.76	1.83E-08
Ptprz1	protein tyrosine phosphatase, receptor z	10.78	6.44E-11
Neural crest			
Snai2	snail homolog 2 (Drosophila)	-6.05	2.87E-10
Erbb3	v-erb-b2 homolog 3	-3.29	2.55E-10
Tcfap2a	transcription factor AP-2, alpha	-4.5	7.04E-07
Twist1	twist1 transcription factor	-7.44	0.00037
Dlx1	distal-less homeobox1	-4.73	0.00022
Dlx2	distal-less homeobox2	-3.67	0.00057
Chondrocyte/osteoblast			
Bgn	biglycan	-11.10	6.92E-11
Mgp	matrix gla protein	-11.35	3.76E-10
Lgals3	galectin	-11.09	2.26E-10
Col2a1	collagen, type II, alpha 1	-0.28	ns
Col9a1	collagen, type IX, alpha1	-0.07	ns
Fmod	fibromodulin	-0.18	ns
Acan	aggrecan	-0.12	ns
Ogn	osteoglycin	-0.29	ns
MSC/pericyte			
Pdgfrb	Pdgf receptor beta	-10.94	2.77E-10
Rgs5	Regulator of G-protein signaling 5	-11.68	9.23E-12
Abcc9	ATP-binding cassette, subfamily C, 9	-9.58	2.17E-11
Ly6a	Lymphocyte antigen 6 comp.; Sca-1	-9.34	7.41E-08
S1pr3	Endothelial diff. spingolipid G-pr. coupl. rec 3	-9.24	2.38E-11
Kcnj8	Potassium inwardly-rect. Channel J8	-8.22	1.08E-08
Enpep	Glutamylaminopeptidase	-6.60	4.63E-05
Spp1	Secreted phosphoprotein 1	-6.87	1.04E-08
Timp2	Tissue inhibitor of metalloprotease 2	-6.23	4.54E-10
CD248	Endosialin	-4.14	5.71E-06

Table 16: Comparison of the gene expression of rNCSCs and pNCSCs analysis of gene expression in rNCSC and BMP NCSC neurospheres by Affymetrix microarrays. The following genes were selected for display: *i)* genes expressed in neural plate epithelial cells *ii)* in the spinal cord ventricular zone, *iii)* in neural crest, *iv)* in mesenchymal neural crest derivatives, *v)* in MSC/perivascular cells. The average signal fold change is shown (log2-fold values). P-values adjusted for multiple hypothesis testing (Benjamin and Hochberg, 1995) (Weber et al., 2015).

Gene ontology of genes differentially expressed in pNCSCs relative to rNCSCs

Cluster	GO term	Fold enrichment	P-value
Annotation cluster 1 (Enrichment score 17.15)			
GOTERM_BP-FAT	GO:0001944~vasculature development	2.49	1.89E-19
GOTERM_BP-FAT	GO:0001568~blood vessel development	2.45	4.01E-18
GOTERM_BP-FAT	GO:0048514~blood vessel morphogenesis	2.48	3.98E-15
GOTERM_BP_FAT	GO:0001525~angiogenesis	2.84	8.17E-15
Annotation cluster 2 (Enrichment score 10,27)			
GOTERM_BP_FAT	GO:0009611~response to wounding	1.98	2.35E-12
GOTERM_BP_FAT	GO:0006952~defense response	1.82	2.45E-10
GOTERM_BP_FAT	GO:0006954~inflammatory response	2.13	2.62E-10
Annotation cluster 3 (Enrichment score 6,28)			
GOTERM_BP_FAT	GO:0001763~morph. of a branch. struct.	2.55	8.82E-11
GOTERM_BP_FAT	GO:0035295~tube development	1.91	8.97E-09
GOTERM_BP_FAT	GO:0048754~branch. morph. of a tube	2.55	2.46E-08
GOTERM_BP_FAT	GO:0048729~tissue morphogenesis	1.81	1.11E-06
GOTERM_BP_FAT	GO:0035239~tube morphogenesis	1.96	1.45E-06
GOTERM_BP_FAT	GO:0060429~epithelium development	1.67	1.41E-05
GOTERM_BP_FAT	GO:0002009~morph. of an epithelium	1.79	5.00E-05
GOTERM_BP_FAT	GO:0060562~epithelial tube morph.	1.93	2.41E-04
Annotation cluster 4 (Enrichment score 6,17)			
GOTERM_BP_FAT	GO:0032963~collagen metabolic process	4.39	9.62E-08
GOTERM_BP_FAT	GO:0044259~multicell. org. m. metab. pr.	4.20	2.34E-07
GOTERM_BP_FAT	GO:0044243~multicell. org. catabolic pr.	4.61	1.11E-06
GOTERM_BP_FAT	GO:0044236~multicell. org. metabolic pr.	3.86	1.12E-06
GOTERM_BP_FAT	GO:0030574~collagen catabolic process	4.52	4.79E-06
Annotation cluster 5 (Enrichment score 5,42)			
GOTERM_BP_FAT	GO:0001501~skeletal system development	1.95	7.74E-10
GOTERM_BP_FAT	GO:0060348~bone development	2.26	6.16E-07
GOTERM_BP_FAT	GO:0001503~ossification	2.24	3.10E-06
GOTERM_BP_FAT	GO:0001649~osteoblast differentiation	2.15	0.00586

Table 17: Gene ontology of genes differentially expressed in pNCSCs relative to rNCSCs. Genes with at least two-fold (q≤0.05) differential expression in pNCSCs vs rNCSCs were analyzed using DAVID (http://david.abcc.ncifcrf.gov). The top five most highly enriched clusters of GO terms are presented. (Weber et al., 2015).

5. Discussion

5.1 DRG-derived NCSCs are reprogrammed to CNS stem cells

NCSCs from E12.5 mouse DRG acquire CNS marker gene expression and downregulate PNS marker genes upon propagation as free floating NS cultures in the presence of EGF and FGF (Binder et al., 2011; Weber et al., 2015). This is reflected by the acquisition of Olig2 in the majority of cells and the loss p75 of expression. Furthermore, rNCSCs show the ability to generate CNS cell types, such as neurons, astrocytes and oligodendrocytes. On the other hand, PNS glial cells or neurons were not detected even after the treatment with factors that promote Schwann cell differentiation in neural crest cultures or after delayed BMP4 application (Binder et al., 2011; Weber et al., 2015). This implies a stable CNS identity of rNCSCs.

The generation of CNS cell types from postmigratory NCSCs was confirmed by findings of the Baron-van-Evercooren lab (2011).The group was able to generate oligodendrocyte-like cells from E10.5 mouse boundary cap-derived cells in vitro and in vivo (Zujovic et al., 2011). Earlier findings also suggested that DRG progenitors are able to generate CNS cell types in vitro (Svenningsen et al., 2004). Furthermore, Dromard et al., (2007) detected Olig2-expressing cells in NS cultures derived from E13.5 DRG that were cultured in the presence of high amounts of FGF and EGF. Dromard and colleagues could not exclude a potential contamination from spinal cord cells, which may result in an overgrowth of CNS-derived stem cells and the loss of NCSCs within a couple of passages. In the present study, the contamination of DRG cultures by CNS cells from the spinal cord was excluded by the following features: *i)* All processes of the dissected DRGs are removed which includes the dorsal roots projections to the spinal cord. *ii)* The starting E12.5 DRG population lacks Olig2-immunoreactive cells, in contrast to E12.5 spinal cord where Olig2 is expressed in the pMN domain (Fu et al., 2002; Zhou et al., 2001). *iii)* Dissected DRGs lack the expression of Olig2, Olig1, Nkx2.2, Nkx6.1 and Pax6, analyzed via qPCR (Binder et al., 2011). *iv)* DRG-

derived NCSCs, cultured with EGF only, could not be maintained beyond passage 2, in contrast to embryonic spinal cord neurosphere cultures. *v)* In contrast to FGF NCSCs that generate oligodendrocyte-like cells with immature morphology, embryonic spinal cord-derived NS differentiate into oligodendrocyte-like cells that display a mature oligodendrocyte morphology.

Since earlier conclusions on the reprogramming of DRG-derived NCSCs were only based on the analysis of differentiated rNCSCs (Binder et al., 2011), it was unknown whether the reprogramming towards CNS identity of rNCSCs under proliferation conditions is complete or partial.

5.1.1 rNCSCs and SCSCs display an identical gene expression profile

To address the issue if CNS reprogramming of rNCSCs is complete, gene expression profiles of 3rd passage rNCSCs and SCSCs were compared by Affymetrix microarrays. The results demonstrate a virtual identical gene expression pattern of rNCSCs and SCSCs. Only one gene is statistically differentially expressed in rNCSCs and SCSCs, but it seems not to play a role in PNS or CNS development. The neural crest and neural crest-derivative marker genes (Tcfap2a, Dlx1, Dlx2, Erbb3, Phox2b, Mgp, Lgals3 and Bgn) are expressed at only background or undetectable levels, indicating that rNCSCs completely lose PNS marker gene expression. On the other hand, CNS restricted genes, which are expressed in the ventricular zone and neuroepithelium, such as Fabp7, Olig1, Olig2, Ptprz1, Tnc, Pax6, Sox3 and Sox1, are expressed at high and comparable levels in rNCSCs and in SCSCs.

Fabp7 is transiently expressed in radial glial cells, just before neural differentation starts (Feng et al., 1994). It is also found in neural stem cells of the postnatal dentate gyrus and plays a role in neurogenesis, by displaying a positive effect on proliferation, and antagonising the survival of new born neurons (Matsumata, et al., 2012). Furthermore it has been reported that Fabp7 is a downstream target of Pax6 (Arai et al., 2005). Pax6 is important during the differentiation of neurons in the CNS, by upregulating

the pro-neural bHLH transcription factor Ngn2. Pax6 also plays a role in proliferating neuroepithelial cells, which suggests that in the early developing cortex Pax6 rather upregulates Fabp7 than Ngn2 (Arai et al., 2005). This leads to the suggestion that Fabp7 and Pax6 are essential for the maintenance of the proliferating state in rNCSCs. The transcription factors Olig1 and Olig2 play a role during oligodendrogenesis. They can act together in a heterodemeric state or alone to regulate tissue-specific development (Meijer et al., 2014). Olig1 and Olig2 are thought to contribute to the oligodendrocyte differentiation of rNCSCs (Binder et al., 2011).

Sox1, Sox2 and Sox3 are SoxB1 transcription factors that bind, as all Sox family transcription factors, to a specific DNA sequence, the so called *Sry* -Box. They often function as binding partners and interact with other Sox transcripion factors to regulate transcription regulatory elements. Sox1, Sox2 and Sox3 play a role during neurulation. Sox2 and Sox3 are already expressed in the neural plate and neural tube (reviewed in Thiel, 2006). In neural progenitor cells SoxB1 transcription factors are thought to be important in the proliferation maintenance and the differentiation repression (Graham et al., 2003). Sox3 is uniformly expressed in rNCSCs but as Sox1 and Sox2 expression was only detected via RT-PCR it remains unclear whether Sox1 and Sox2 are expressed in all or only subpopulations of rNCSCs. Those three transcription factors could contribute to the maintenance of the proliferative and undifferentiated state of rNCSCs.

Tenascin c (Tnc) is a glycoprotein which is expressed during early stages of CNS development and is downregulated during CNS maturation. It remains expressed in at least two stem cell niches, the gyrus dentatus and SVZ. In the cortex and SVZ of Tnc-deficient postnatal mice it has been shown that progentitor cell proliferation is reduced and that OPC proliferation is impaired (Garcion et al., 2001). This implies that Tnc is essential for oligodendrocyte formation in rNCSCs. In addition, Ptprz1 and Tnc are carriers of the chondroitin sulphate proteoglycan (CH-PG) recognized by the mAB 473HD and the LewisX (LeX) glycan recognized by the mABs 5750^{Lex}, which are uniformly expressed in rNCSCs and SCSCs. CH-PG is expressed in neural progenitor

cells of the embryonic forebrain, and when CH-PG is digested in cortical precursor cells NS formation fails. CH-PG interacts with FGF, thus playing an important role in NS formation, self-renewal and proliferation (Ito et al., 2005; Sirko et al., 2010). 5750^{Lex} is expressed in the developing embryonic spinal cord and coexpressed with Nestin-positive neural precursor cells at E13.5 (Karus et al., 2013).

Taken together, these results suggest that rNCSCs acquire a complete SCSC CNS identity, demonstrated by the identical gene expression pattern of rNCSCs and SCSCs, the downregulation of PNS marker genes and the uniform expression of the CNS markers Olig2, Sox3, Fabp7, 473HD and 5750^{Lex}.

5.1.2 rNCSCs acquire a ventral neural tube identity

Some of the CNS marker genes expressed in rNCSCs and SCSCs, such as Sox2, Sox3 and Zic1, are not only expressed in spinal cord and brain but additionally expressed at earlier stages in the neural plate (reviewed in Thiel et al., 2006; Nagai et al., 1997). This raises the question whether rNCSCs have a spinal cord/brain or neural plate identity. The data set of this study demonstrates that rNCSCs have a spinal cord rather than neural plate identity, since rNCSCs lack Oct4 expression that is expressed during neural plate development (Archer et al., 2011). Furthermore, Olig2, Fabp7 and Pax6 are expressed in the neural tube and brain but are not yet detectable in the neural plate (Feng et al., 1999; Bertrand et al., 2000; Novitch et al., 2001; Binder et al., 2011). The acquisistion of a spinal cord identity of rNCSCs was already suggested in an earlier published data set (Binder et al., 2011). It was shown that rNCSCs express trunk Hox genes (Hoxb6 and Hoxc10) as well as ventral neural tube marker genes (Olig2 and Nkx2.2). A brain identity of rNCSCs was excluded because cortical markers were not upregulated in rNCSCs (Binder et al., 2011). Several findings of the present study provide additional support for the assumption that rNCSCs acquire a spinal cord identity. *i)* rNCSCs and SCSCs have an identical gene expression pattern. *ii)* rNCSCs and SCSCs express trunk (Hoxb6 and Hoxc10) marker genes and lack fore/

midbrain markers (Emx1, Nr2e1 and Foxg1) (Kelly et al., 2009; Bishop et al., 2001). *iii)* Unsupervised principal component analysis (PCA) of the microarray data sets of rNCSCs and brain derived stem cells such as granule neuron progenitors (GNPs) and radial glia progenitors (RGPs) demonstrate that rNCSCs and brain-derived stem cells do not share common gene expression characteristics.

PCA analysis also shows that all three probe sets of rNCSCs cluster together which demonstrates the homogeneity and reproducibility of rNCSC cultures. These data support and extend the findings of Binder et al., (2011) using a genome wide expression analysis to demonstrate that rNCSCs acquire the identity of SCSCs.

5.1.3 rNCSCs are directly reprogrammed to cells with SCSC identity

Two mechanisms for cell lineage conversion that are very well defined are termed “reprogramming (with pluripotency state)” and “direct reprogramming or transdifferentiation”. The question arises if the reprogramming process of rNCSCs involves a transient pluripotency state.

i) Reprogramming: It has been shown for the first time in 2006 that the combination of four transcription factors (Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M) (OSKM)) induces the reprogramming “cascade” towards pluripotency when they are ectopicially overexpressed in differentiated fibroblasts (Takahashi et al., 2006). These induced pluripotent stem cells (iPSCs) show the potential to give rise to cells of all three germ layers.

The reprogramming involves a number of intermediate states. The initial steps of the reprogramming towards pluripotency, before transcriptional changes take place and cell division starts, include epigenetic modifications i.e. remodeling chromatin structure, DNA methylation and Histone modifications. The core of three pluripotency inducing genes, OSK, shows the ability to re-open the chromatin. Furthermore, it has been demonstrated that OSK access and bind to genomic DNA (regulatory DNA sites) that are fully packed in nucleosomes. The opening of the chromatin is important to allow the

transcription factors to bind on recognition elements, such as specific enhancers and promotor regions of the DNA, to initiate the transcription of their target genes. c-Myc enhances the initial binding of OSK but cannot access target sites on his own. c-Myc is also not required for the reprogramming towards pluripotency. Interestingly, most pluripotency restricted genes are activated late during the reprogramming process. This is due to the fact that their promotor regions are highly methylated and demethylation starts late during the reprogramming process. On the other hand somatic enhancers that are inactivated by the reprogramming factors are lately hypermethylated during this process. Since the reprogramming towards pluripotency requires a complex “cascade” of changes it is not surprising that it only occurs at a low frequency in below 1 percent of cells within a time period of 1 to 2 weeks (reviewed in Papp and Path, 2013).

ii) Direct reprogramming or transdifferentiation: Another mechanism of cell fate modification, termed direct reprogramming or transdifferentiation, is the direct change or switch of a differentiated cell type into another differentiated cell type, without going through a pluripotency state (reviewed in Amamoto and Arlotta, 2014). This process involves cell dedifferentiation to an intermediate state, that can include cell cycle reentering and subsequently differentiation to a new cell lineage. Transdifferentiation or direct reprogramming of cells can also include an intermediate unnatural phase where two genetic programs are activated (reviewed in Jopling et al., 2011). One example for the direct reprogramming is the generation of induced neural stem cells (iNSCs) from fibroblasts by ectoptic expression of defined factors (Han et al., 2012).

In this study it was shown that NCSCs directly reprogram into rNCSCs that have a SCSC identity. Reprogramming that includes a pluripotency state is excluded because passage 1 and passage 3 rNCSCs NS express pluripotency genes only at background or undetectable levels. Furthermore, the results imply that the direct reprogramming involves dedifferentiation, the loss of PNS characteristics into an intermediate state that includes cell cycle reentering. An unnatural state where two genetic programms are activated is excluded because passage 1 rNCSCs lack cells coexpressing PNS and CNS markers (Olig2/p75). Thus, the results suggest the loss of one program by

dedifferentiation (PNS identity) and subsequently the upregulation of another program (CNS identity). The absence of a pluripotent state during rNCSC reprogramming is also supported by previous results that rNCSCs do not give rise to tumors upon the transplantation into embryonic or adult mouse brains (Binder et al., 2011). This is a considerable advantage as compared to iPSCs and ESCs that generate teratomas after transplantation into syngeneic or immunodeficient mice (reviewed in Ben-David and Benvenisty, 2011). Another disadvantage of iPSCs is the retrovirally introduced transgenes. At the present stage iPSCs cannot be used for stem cell based therapy because of safety concerns (Aoi et al., 2008). Since rNCSCs were generated without genetic modifications and give rise to CNS progeny, NCSCs are a safe potential source for regenerative medicine in the CNS.

5.1.4 Direct reprogramming of BMP NCSCs into oligodendrocyte-like cells by Olig2 overexpression

It has been demonstrated that forced expression of the combination of Olig2 and Sox10 induces oligodendrocyte marker gene expression in embryonic fibroblasts by direct reprogramming (Najm et al., 2013; Yang et al., 2013). This raises the question if ectopic overexpression of Olig2 in BMP NCSCs that endogenously express Sox10 is sufficient to induce oligodendrogenesis. For forced Olig2 expression in BMP NCSCs, a *Piggy Bac* transposon plasmid containing *mmOlig2* was generated. *Piggy Bac* transposons integrate into the host genome but can be removed at a later stage by the transfection of the cells with a transposase-expressing vector. The transient transgene introduction via the *Piggy Bac* transposon avoids permanent genetic alterations, in contrast to the permanent retrovirally introduced genes, for example the retrovirally introduced pluripotency genes in iPSCs (reviewed in Ben-David and Benvenisty, 2011). Ectopic overexpression of Olig2 in BMP NCSCs, which express Sox10 at high levels, induces the oligodendrocyte marker O4 in a very small fraction of Olig2-positive cells. O4/Olig2-coexpressing cells display an oligodendrocyte-specific morphology, which

implies that Olig2 overexpression is able to induce the direct reprogramming towards oligodendrocyte lineage, albeit in a very low number of cells. The low number of induced oligodendrocyte-like cells by ectopic overexpression of Olig2 is in agreement with the findings of Njam et al., (2013). Njam and colleagues demonstrated that the ectopic overexpression of Olig2 and Sox10 induces PLP1 expression in embryonic fibroblasts with lower efficiency than the combination of Olig2, Sox10 and Nkx6.2.

The low efficiency of oligodendrocyte generation may also be explained by effects of the cellular context on Olig2 phosphorylation. The transcription factor Olig2 exists in a phosphorylated and unphosphorylated state (Sun et al., 2011; Meijer et al., 2014). In cycling precursor cells Olig2 is phosphorylated at triple serin motifs within the terminal domain. During the differentiation into cells of the oligodendrocyte lineage the phosphorylation vanishes (Sun et al., 2011; Meijer et al., 2014). Thus, the Olig2 protein may be phosphorylated in the majority of Olig2-expressing cells which would block the differentiation into oligodendrocytes. Taken together, these preliminary results demonstrate the possibility to induce oligodendrocyte development in BMP NCSCs by transdifferentiation. These findings must be confirmed by additional experiments and marker genes for oligodendrocytes as well as a more efficient combination of induced transcription factors. In addition, it remains to be shown that the oligodendrocyte development is possible after the removal of the introduced genes.

5.2 The neural crest fate is maintained in the presence of BMP4

rNCSCs from E12.5 DRG acquire a SCSC identity in the presence of EGF and FGF. The presence of BMP4 in a proliferation medium (BMP4/EGF/FGF) prevents NCSCs from turning into SCSCs and furthermore it maintains the neural crest identity in BMP NCSCs. The reprogramming towards CNS identity is already efficiently antagonized by BMP4 at passage 1, reflected by the lack of Olig2 and the expression of p75. NCSCs are generally considered to coexpress p75 and Sox10 (Wong et al., 2006; Stemple and Anderson, 1992; Paratore et al, 2001). The large fraction of cells devoid of both,

Sox10 and p75, is explained by the presence of neural crest-derived mesenchymal stem cells, as discussed below (see section 5.2.2). Sox10-positive but p75-negative cells in BMP NCSCs may represent PNS glial cells that have down regulated p75 in culture. Downregulation of p75 in glial cells of the DRG has been observed during embryonic development (Hu et al., 2011). Functional evidence for the maintenance of neural crest fates was obtained by the generation of PNS glia and neurons in BMP NCSC cultures. BMP NCSCs differentiate into Schwann cells in response to NRG1 and forskolin, which was shown to induce Schwann cell differentiation in neural crest cultures (Wolpowitz et al., 2000; Paratore et al., 2001; Morrissey et al., 1995; Yamada et al., 1995). Furthermore, Tuj1/p75- and Tuj1/Phox2b-coexpressing neurons were detected. The expression of the autonomic neuron marker Phox2b suggests that autonomic neurons are generated from DRG-derived BMP NCSCs. This is not unexpected as sympathetic neuron development involves the induction of Phox2b by BMP4 (Schneider et al., 1999; Reissmann et al., 1996; Shah et al., 1996; reviewed in Rohrer, 2011). However, additional expression of genes characteristic for sympathetic neurons (TH) were not observed under the conditions used. A small fraction of Tuj1-expressing neurons induce the PNS neuron marker Peripherin after the differentiation in the presence of neurotrophins (Toy et al., 1990). Of note, Peripherin is only expressed in a subpopulation of postmitotic neurons of the DRG (Toy et al., 1990). Therefore, it cannot be excluded that Tuj1-positive but Peripherin-negative neurons are also peripheral postmitotic neurons. Melanocytes, an additional neural crest progeny, were undetectable under the medium conditions used. The presence of melanocyte progeny in NCSC cultures varies between different cultures/sources (Morrison et al., 1999; Sieber-Blum and Cohen, 1980; Bronner-Fraser et al., 1980; Bronner-Fraser and Fraser, 1989; Amoh et al., 2005; Sieber-Blum and Grim, 2004) and may be dependent on the activation of Wnt signaling (reviewed in Delfíno-Machin et al., 2007).

These results demonstrate that in the presence of BMP4 neural crest markers are partially maintained and the upregulation of the CNS marker gene Olig2 is prevented. The neural crest identity is demonstrated by the ability to differentiate into neural crest

progeny.

5.2.1 The gene expression profile indicates high expression of neural crest-specific genes in BMP NCSCs

To adress the issue whether the reprogramming towards CNS identity is fully blocked in the presence of BMP4/FGF/EGF, gene expression profiles from 3rd passage BMP NCSCs and rNCSCs were obtained. BMP NCSCs and rNCSCs display strong differences in gene expression pattern. CNS specific genes such as Fabp7, Olig1, Olig2, Ptprz1, Tnc, Pax6, Sox3 and Sox1 are restricted to rNCSCs and are only expressed at background or undetectable levels in BMP NCSCs. BMP NCSCs express genes characteristic for neural crest and neural crest derivatives, such as Snai2, Erbb3, Sox10, Dlx1/2 and Tcfap2a/b, at high levels.

Snai2 is one of the early neural crest specifiers. Of note, it has been suggested that BMPs positively regulate Snai2 expression by the direct binding of Smad to the Snai2 promotor (Sakai et al., 2005). In addition, Snai2 can be upregulated in neural plate explants of chicken embryos by BMPs (Liu and Jessel, 1998; Marchant et al., 1998). This implies that Snai2 upregulation in BMP NCSCs may be mediated by BMP4.

Sox10 is expressed in migrating neural crest cells and is down regulated in early neurons. It is maintained in the Schwann cell lineage, plays a role in early stages of Schwann cell development and later during the differentiation into myelinating Schwann cells (Britsch et al., 2001; Schreiner et al., 2007). Furthermore, it is suggested that Sox10 plays a crucial role in the maintenance of the stem cell state of NCSCs (Kim et al., 2003).

Erbb3 is a receptor tyrosine kinase and belongs to the EGF receptor family. ErbB3/ErbB2 receptors are important for the survival and development of Schwann cell precursor cells (Wolpowitz et al., 2000; Morrissey et al., 1995). Interestingly, both, Sox10 and Erbb3, expression is initiated just when the neural crest cells delaminate. It has been shown that Sox10 positively regulates Erbb3 expression and directly modulates Erbb3

transcription in the zebrafish neural crest (Britsch et al., 2001; Prasad et al., 2011). The Erbb3 knock out causes defects of neural crest derivatives, including the loss of peripheral glial cells (Erickson et al., 1997; Wolpowitz et al., 2000). High expression of Erbb3 in BMP NCSCs supports the suggestion that Sox10-positive but p75-negative cells are associated with PNS glial cells as already discussed above.

Tcfap2a is expressed in premigrating and migrating neural crest cells (reviewed in Hilger-Eversheim et al., 2000). In the zebrafish it was shown that Tcfap2a directly activates Sox10 expression in the neural crest (Van Otterloo et al., 2012). Additionally, in Tcfap2a-deficient mice the neural crest migration is impaired, which results in abnormalties of neural crest derivatives (Schorle et al., 1996; Zhang et al., 1996). Tcfap2a is also expressed in Schwann cell precursor cells but is down regulated during differentiation (reviewed in Jessen and Mirsky, 2005). Notably, a high fraction of approximately 79% of BMP NCSCs express Tcfap2a. Tcfap2b, as Tcfap2a, is expressed in the neural crest and in neural crest derivatives, such as the sympathetic ganglia, DRG and chromaffin cells of the adrenal gland (Moser et al., 1997; Hong et al., 2011, Schmidt et al., 2011).

The results indicate that BMP4 in combination with EGF and FGF is responsible for the neural crest maintenance and the inihibition of CNS reprogramming of E12.5 DRG-derived NCSCs.

5.2.2 The DRG displays a source for MSC-like/pericyte precursor cells

Interestingly, a strong enrichment of genes, such as Bgn, Mgp, Lgals3, Col2a1, Col9a1, Fmod and Acan, expressed by mesenchymal derivatives, was detected via Affymetrix microarray in BMP NCSCs. These genes contribute to the development of chondrocytes and osteocytes. Mesenchymal neural crest derivatives are not generated by the trunk neural crest in higher verterbrates. In contrast, the cranial neural crest gives rise to the facial skeleton and other cranial mesenchymal tissues, including smooth muscle, adipose tissue of skin over the calvarium, skin in the face as well as the ventral part of

the neck and many connective components in cephalic organs (reviewed in Le Douarin et al., 2004). Trunk mesenchymal derivatives are only found in lower vertebrates, where the dorsal fin is neural crest-derived (Kague et al., 2012), suggesting that the potential of trunk NCSCs to generate mesenchymal derivatives in vivo is lost during evolution. In the present study it is shown that the mesenchymal potential of trunk neural crest can be induced in vitro by defined medium conditions (BMP4/EGF/FGF). These findings are in agreement with the findings by John et al., (2011), who demonstrated TGF-β-dependent generation of mesenchymal derivatives from trunk-derived NCSCs of the pharyngeal apparatus. Sox10 was identified as a key player in the transition of trunk NCSCs into mesenchymal derivatives. Sox10-positive cells are unable to differentiate into osteocytes or chondrocytes. The mesenchymal potential of trunk neural crest cells is induced by Sox10 downregulation, mediated by TGF-β1 (John et al., 2011). BMP4 is a member of the TGF-β family. The findings of the present study indicate that BMP4, as TGF-β1, may induce mesenchymal fates in NCSCs and that the downregulation of Sox10 during NS culture in the presence of BMP4 elicits the mesenchymal potential. The expression of trunk marker genes, such as Hoxb6 and Hoxb10 (Data not shown), in BMP NCSCs excludes a switch of trunk to cranial identity of BMP NCSCs.

Surprisingly, genes expressed in mesenchymal stem cells (MSCs) and pericyte progenitors, such as Pdgfrb, Rgs5, S1pr3, Acta2, Spp1, Timp1, CD248, Kcnj8, Abcc9 and Ly6a, were additionally enriched in BMP NCSCs. As MSCs and pericytes both in vivo and in culture share common marker gene expression, it is not easy to distinguish these cell types (Crisan et al., 2008; Schwab et al., 2007). Furthermore, the origin and identity of MSCs in native tissues is poorly understood. Some research groups suggest that all or at least a subpopulation of MSCs are pericytes (reviewed in Caplan, 2008; Blocki et al., 2013). Notably, in the cranial region, cells of the neural crest generate pericytes in face and neck region as well as in the meninges (Etchevers et al., 1999; Etchevers et al., 2001; reviewed in Armulik et al., 2011). These data suggest that the addition of BMP4 to the proliferation medium promotes MSC/pericyte progenitor generation in BMP NCSCs. Since pericytes, as MSCs and the cranial neural crest, show

the potential to differentiate into osteocytes, chondrocytes and adipocytes (Crisan et al., 2008), an enrichment of osteocyte and chondrocyte marker expression could be associated with the differentiation potential of pericytes/MSCs in BMP NCSCs. To further prove the potential of trunk derived BMP NCSCs to generate mesenchymal progeny cells, the differentiation into mature chondrocytes, osteocytes and adipocytes must be demonstrated. These experiments could not be accomplished within the time frame of this study.

In conclusion, BMP NCSCs represent a heterogeneous cell population which consists of neural crest cells with a neural potential that represents the Sox10-positive fraction as well as neural crest-derived MSCs/pericytes and mesenchymal progeny that is expected to represent the Sox10-negative fraction.

5.3 Adult NCSCs maintain neural crest fates

For regenerative medicine, NCSCs must be isolated from an adult, easily accessible tissue source. Since the DRG does not fulfill these criteria, the palate of adult mice was used in this study to isolate NCSCs. Within the adult rat palate p75- and Nestin-positive cells reside in the innervation of Meissner corpuscules and Merkel cells. It is thought that these cells generate NS in palatal cultures (Widera et al., 2009; Widera et al., 2011). In the present study NCSCs from the adult mouse palate (pNCSCs) were isolated and cultured in the presence of EGF and FGF. The data set demonstrates that NS-forming cells arise from Sox10-positive NCSCs as discussed below. NS cells were analyzed for the expression of NCSC marker genes. A continuous decrease in the proportion of Sox10- and p75-positive cells is observed until passage 3, the latest passage documented. This correlates with the observed decrease of Sox10 and p75 in rNCSCs derived from E12.5 DRG (Binder et al., 2011). However, the CNS marker gene Olig2 was not induced in pNCSCs. The microarray data of 3rd passage pNCSCs display an enrichment of neural crest marker genes and pericyte/MSC marker gene expression in pNCSCs. As pericytes in the face and neck region as well as the vast

majority of palatal mesenchymal cells are derived from the cranial neural crest (Ito et al., 2003), the expression of pericyte/MSC marker genes in pNCSCs reflects the normal potential of the cranial neural crest. This suggests that MSC/pericyte marker genes are upregulated in the large fraction of Sox10-negative cells and that their expression correlates with the down regulation of Sox10.

Why are pNCSCs not able to induce CNS traits, such as Olig2 expression even though they are cultivated under nearly the same conditions as embryonic and postnatal DRG-derived NCSCs? *i)* Proliferation medium of pNCSCs is supplemented with higher amounts of FGF and the cells were cultured under low oxygen conditions. Under the same medium conditions, used for rNCSC propagation, pNCSCs could not be propagated as NS culture. The different culture condition could prevent the acquisition of CNS traits. On the other hand, rNCSCs can be propagated as NS culture and induce Olig2 expression under the same medium condition which were used for pNCSCs (Data not shown), concluding that the different culture conditions can be excluded as explanation. *ii)* The inability of pNCSCs to generate CNS progeny could also be explained by the reduced plasticity of NCSCs at adult stages. This notion is supported by the finding that postnatal DRG NCSCs only partly lose the PNS differentiation potential, whereas embryonic NCSCs are completely reprogrammed to CNS identity. *iii)* The high level BMP production by pNCSCs (Data not shown) may be responsible for the differentiation of pNCSCs towards mesenchyme/pericytes rather than neural development. The decrease of Sox10 expression to about 10% of pNCSCs can not be explained exclusively by BMP signaling as BMP NCSCs maintain Sox10 and the PNS potential even in the continuous presence of BMP4. *iv)* An alternative explanation, discussed below in detail, would be different cell identities of embryonic and adult NCSCs.

5.4 Identity and origin of NCSCs at different postmigratory locations

Why show NCSCs isolated from different postmigratory tissues and developmental stages differences in their developmental potential?

During embryonic development the neural crest cells delaminate and migrate to different locations within the entire embryo. Along the anterioposterior (AP) axis all neural crest cells in the trunk region have the same developmental potential in vivo (Le Dourian, 1982; Le Douarin and Kalcheim, 1999). The fate of neural crest cells is determined during the migration to their destinations by different cues in the environment (reviewed in Gilbert, 2000). In addition, there is a difference in the developmental potential of cranial and trunk neural crest cells with respect to mesenchymal fates. Cell lineage tracing experiments, using *Wnt1*, *Sox10* and *P0* reporter mouse lines, demonstrated that the cranial neural crest gives rise, besides neural progeny and melanocytes, to mesenchymal derivatives of the facial skull and facial mesenchymal tissue (reviewed in Dupin and Douarin, 2014). On the other hand, trunk derived neural crest cells have lost the ability to generate mesenchymal derivatives and are restricted to give rise to neural progeny as well as dorsally located melanocytes. NCSCs from varius postmigratory regions display a different developmental potential in vitro and upon transplantation. It is not really surprising that cranial postmigratory NCSCs in vitro have a broader developmental potential, by generating mesenchymal progeny, than trunk postmigratory NCSCs (reviewed in Kaltschmidt et al., 2011). But also the developmental potential of postmigratory trunk NCSCs differs depending on their location. Sciatic nerve-derived NCSCs differentiate into glial cells rather than into neurons upon transplantation into the gut. They are unable to populate the gut or even to differentiate into enteric neurons, whereas gut derived NCSCs differentiate into enteric neurons (reviewed in Delfino-Machín et al., 2007). The distinct developmental potential of postmigratoty NCSCs, isolated by different groups and in different locations, may be explained by different propagation protocols but may also be due to different cellular identity of the “neural crest stem cells”. Notably, there is no set of markers that

allows to unambiguously identify neural crest stem cells. Commonly used markers, such as p75, Sox10 and Nestin, are expressed in NCSCs but also in glial cells (Kruger et al., 2002; Sieber-Blum et al., 2004; reviewed in Delfino-Machìn et al., 2007). Thus, to identify a neural crest stem cell rather than glial origin of isolated cells, glial markers must be absent. Morrison et al., (1999) identified neural crest stem cells from the sciatic nerve by the expression of p75 and the lack of glial marker P0, but in most studies the analysis of isolated stem cells for glial marker gene expression is neglected.

Also for rNCSCs from embryonic DRG that arise from Sox10-positive cells (Binder et al., 2011), it remains unclear whether they originate from Sox10-positive glial cells or residental neural crest stem cells. Therefore, they were termed reprogrammed neural crest-derived stem cells, pointing out that a glial origin is not excluded. A glial origin of propagated NS from the embryonic chick DRG has previously been demonstrated (Ellen Binder, Phd thesis, 2009; unpublished Data). It was shown by cell sorting that proliferating cells with stem cell characteristics from the DRG of chicken embryos are derived from O4-positive glial cells. Neurosphere-forming cells from the adult rat DRG were also shown to arise from satellite glial cells (Li et al., 2007).

Within the adult rat palate, p75- and Nestin-positive cells reside in the innervation of Meissner corpuscules and Merkel cells. It is thought that these cells give rise to NS when the adult palate is dissociated and cultivated with EGF and FGF (Widera et al., 2009; Widera et al., 2011). In the present study, a *Sox10-GFP* mouse line was used to identify NS-forming cells from the mouse palate. The finding that 95% of the NS contain GFP-positive cells after 5 days in culture strongly suggests that pNCSC NS arise from Sox10-positive neural crest-derived stem cells. A glial origin of pNCSCs is suggested because in freshly isolated palatal cell cultures Sox10-positive cells display a Schwann cell morphology. Notably, the proportion of Sox10 (GFP)-positive NS decreases rapidly in culture, which may either be explained by decreased Sox10 expression or by an overgrowth of NS that arise from a different cell type, such as pericyte. The generation of pNCSCs from pericytes with neural crest origin could explain the different developmental potential of pNCSCs as compared to rNCSCs that

arise from Sox10-positive cells. Another, more likely possibility is that pNCSCs are derived from Sox10-positive cells that acquire a more restricted developmental potential during development. This is supported by the findings of other research groups that have demonstrated that NCSCs within aging show a more restricted developmental potential (reviewed in Delfino-Machìn et al., 2007). A NCSC origin of pNCSCs is also supported by the findings that pericytes from the cortex, that are neural crest-derived (Etchevers et al., 2001), lack the expression of Sox2 (Karow et al., 2012), which is strongly expressed by pNCSCs as well as by immature Schwann cells (Aquino et al., 2006).

In conclusion it remains unclear whether the heterogeneous potential of postmigratory NCSCs is caused by different cell type origin, different propagation protocols or through a restriction in the developmental potential during NCSC development in the specific tissue.

In order to compare the developmental potential of different postmigratory NCSCs the cell type origin of NCSCs must be clarified by the establishment of a uniform isolation protocol and furthermore the propagation protocols must be standardized. *i)* The Cell type origin of NCSCs must be identified by cell sorting via neural crest marker genes, such as Nestin, Sox10 or p75. *ii)* Since there is no marker gene specific for NCSCs yet, cells must be at least negative for glial markers, such as P0, O4 or PLP. *iii)* An uniform propagation protocol must be established.

5.5 The role of FGF and BMP4 in the reprogramming process towards CNS identity of rNCSCs

In the early development of the organism, BMPs are involved in the neural crest induction. Intermediate levels of BMP signaling, resulting from the presence of BMP inhibitors, lead to the induction of the neural crest. However, high FGF signaling induces the neural plate, and it is proposed that FGFs contribute to the adjustment of BMP signaling involved in neural crest induction (reviewed in Meulemans and Bronner-Fraser, 2004;

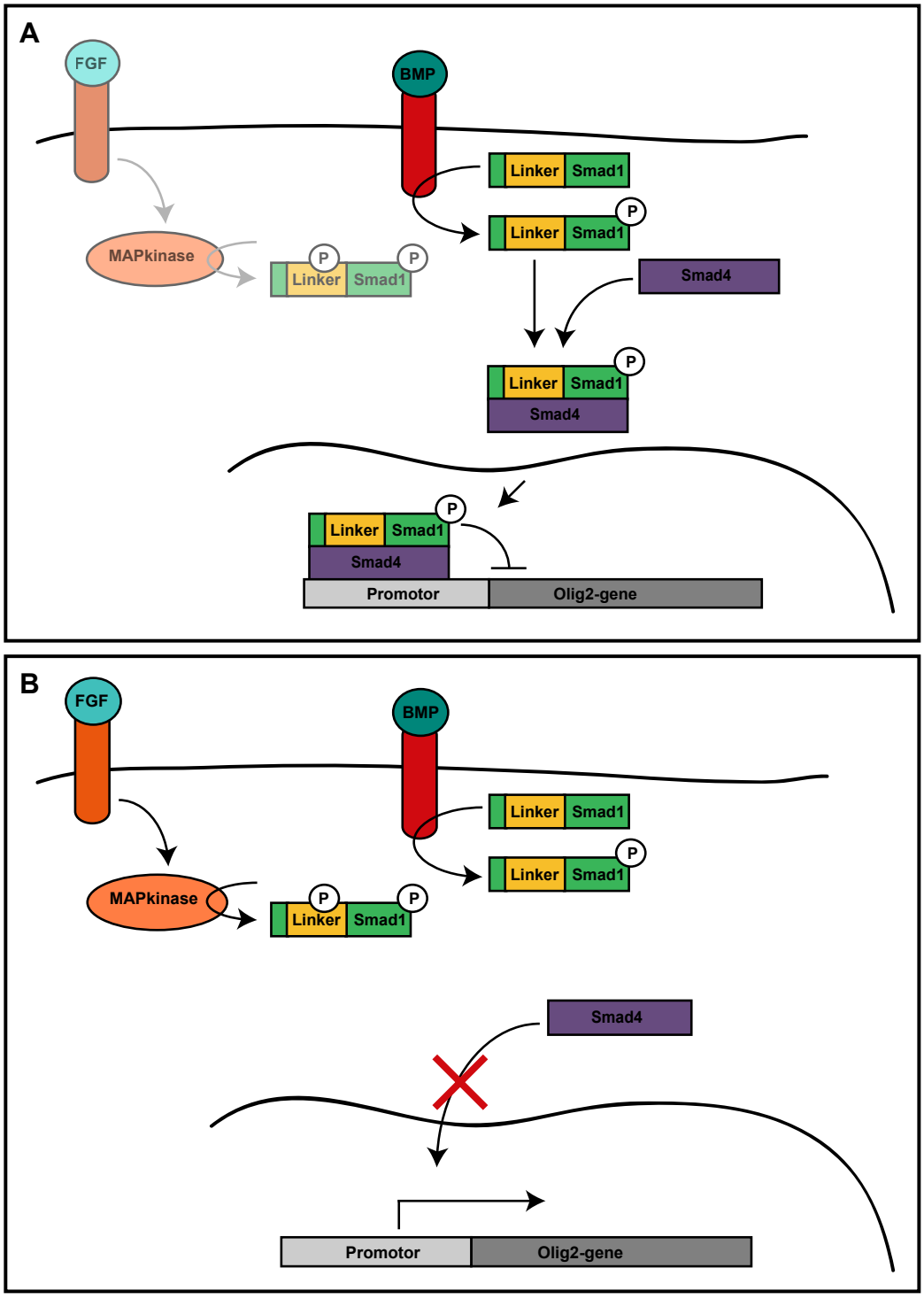

Figure 27: Two possible pathways that may play a role in the induction (rNCSCs) or repression (BMP NCSCs) of Olig2. (A) In BMP NCSCs, the FGF MAPkinase (MAPk) pathway and BMP Smad dependent pathway stay in competition. BMP Smad dependent pathway (bright colors) has a dominant effect over FGF MAPk pathway (pale colors). This results in the dimerization of Smad1 and Smad4 and the transition into the nucleus. Subsequently, Smad4 is able to bind to the Olig2 promotor to inhibit Olig2 transcription. (B) rNCSCs are cultured in the presence of FGF. In response to FGF, the Linker domain of Smad1 is phosphorylated through the MAPk pathway. This leads to the inhibition of the dimerization of Smad1 and Smad4. Therefore, Smad4 has lost the ability for transnuclear migration to inhibit Olig2 transcription.

Streit et al., 2000; Kudoh et al., 2004; La Bonne and Bronner-Fraser, 1998). The BMP-dependent neural crest induction also indicates that BMPs prevent the induction of a CNS identity at this stage. The antagonism between BMP and FGF in the generation of a neural crest and CNS identity also explains the findings in NCSC cultures. In cultured BMP NCSCs, FGF may contribute to the intermediate level of BMPs and leads to the neural crest maintenance. High FGF signaling in the absence of BMP is thought to induce CNS fates in rNCSCs.

What are the molecular mechanisms that underlie the antagonism between BMP and FGF? BMPs belong to the TGF-β family that bind to cell surface serine/threonine kinase receptors and induce the dimerization of Co-Smad (Smad4) and R-Smad (Smad1,5,8) in the cytoplasm. Subsequently, the expression of the inhibitor of differentiation (Id) family is induced in the nucleus. The Id family consists of basic helix-loop-helix (bHLH) transcription factors, such as Id1-Id4, that do not contain the basic DNA binding region adjacent to the bHLH region. Ids are known to inhibit transcription by dimerization with HLH transcription activators, which then are unable to bind to the DNA.

On the other hand, FGF is able to repress the canonical Smad-dependent pathway of BMPs through the phosphorylation of Smad1 on the Linker domain. As a result, phosphorylated Smad1 prevents the dimer formation with Smad4, impairing the BMP Smad-dependent pathway (reviewed in Miyazono et al., 2005). Reduced Smad signaling results in the downregulation of Id genes which leads to the induction of neuronal differentiation as it was reported for ESCs (reviewed in Zhang and Li, 2005). The specific steps resulting in CNS versus PNS/neural crest development downstream of BMP/FGF are not understood. In the following section potential mechanisms involved in the control of Olig2 expression are discussed as exemplary model for the BMP/FGF antagonism.

In vivo and in vitro evidence shows that BMPs inhibit Olig2 whereas FGFs induce Olig2 in CNS cultures (Bilican et al., 2008; Chandran et al., 2003; Mekki-Dauriac et al., 2002). Previous findings demonstrate that Smad4, one of the Co-Smads, directly regulates the Olig2 promotor. When Smad4 binding on the promotor is prevented,

high expression of Olig2 is observed. This suggests that BMPs directly regulate Olig2 expression (Bilican et al., 2008). It is also proposed that FGF signaling through MAPk-pathway contributes to relieved Olig2 repression by intefering with Smad1 and Smad4 dimerization. This prevents the binding of Smad4 to the Olig2 promotor (Blilican et al., 2008).

The present study indicates that FGF may be able to induce Olig2 expression in neurosphere cultures of rNCSCs by inhibiting the Smad4 binding on the Olig2 promotor (Figure 27). Notably, BMPs are expressed in the DRG during development (Guha et al., 2003) and also in pNCSCs.

On the other hand, high BMP4 signaling in neurosphere cultures of BMP NCSCs may inhibits Olig2 expression by Smad4 binding on the Olig2 promotor which leads to the inhibition of Olig2 transcription (Figure 27).

Taken together, it is hypothesized that the CNS versus PNS/neural crest identity of NCSCs is controlled by the antagonistic function of BMP and FGF.

6. Summary

The neural crest gives rise to the neurons and glial cells of the peripheral nervous system (PNS) (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). Self-renewing neural crest-derived stem cells (NCSCs) are present in migratory neural crest and various postmigratory locations, including peripheral ganglia (Duff et al., 1992; Morrison et al., 1999; Kruger er al., 2002). It is demonstrated that NCSCs from embryonic mouse dorsal root ganglia (DRG) are reprogrammed in neurosphere (NS) cultures in the presence of EGF and FGF. Reprogrammed NCSCs (rNCSCs) generate exclusively central nervous system (CNS) progeny, both in vitro and upon transplantation into the mouse brain (Binder et al., 2011). In this study the timing and mechanisms underlying the reprogramming were addressed. Most of the cells acquire CNS characteristics at passage 2, reaching a stable proportion of >90% of Olig2-positive cells at passage 3, which is maintained at least up to passage 10. The PNS marker p75 is completely lacking from passage 3 onwards. Furthermore, it was shown that the reprogramming does not involve a transient pluripotency state. This suggests a direct reprogramming of NCSCs to cells with CNS identity. The reprogramming leads to a stable CNS identity as shown by delayed BMP4 application. This result is in agreement with the previous observation that rNCSCs only generate CNS progeny, in particular mature myelinating oligodendrocytes, upon transplantation into embryonic, postnatal and lesioned adult mouse brains (Binder et al., 2011). Genome wide gene expression profiles of rNCSC NS demonstrates already in culture a complete switch to a (spinal cord stem cell) SCSC CNS identity. These results demonstrate a complete reprogramming of PNS progenitors to CNS identity without genetic modification and imply PNS cells as a source for stem cell-based CNS therapy.

The reprogramming of NCSCs is completely blocked in the presence of BMP4 in NS cultures, as shown by the expression of neural crest markers p75 and Sox10. In addition, BMP4 NCSCs generate PNS neurons (Tuj1/Phox2b- and Peripherin/Tuj1-coexpressing cells) and Schwann cells (O4/p75-coexpressing cells). Genome wide

gene expression profiles of BMP NCSCs demonstrate that BMP NCSCs express genes at high levels which are characteristic for neural crest/neural crest derivatives, mesenchymal derivatives of neural crest and perivascular pericytes/MSCs. On the other hand CNS marker genes are restricted to rNCSCs and are only expressed at background or undetectable levels in BMP NCSCs. These findings imply that the CNS versus PNS identity is controlled by antagonistic functions of FGF and BMP4.

The use of rNCSCs for cell therapies requires an accessible source of these cells in the adult organism. Since the DRG is not an easily approachable tissue source, the adult mouse palate, containing NCSCs, was chosen. These results suggest that pNCSCs arise from Sox10-positive neural crest-derived stem cells, that downregulate PNS marker gene expression, such as Sox10 and p75, in NS culture. Contrary to rNCSCs, CNS marker upregulation was not observed. Notably, genome wide gene expression profiles of pNCSCs demonstrate an enrichment of genes expressed by mesenchymal derivatives and perivascular pericytes/mesenchymal stem cells. Since the cranial crest gives rise, besides PNS neural progeny and melanocytes, to mesenchymal derivatives, the results demonstrate that pNCSCs have a restricted developmental potential in comparison to rNCSCs and acquire mostly normal fates of the cranial neural crest.

Taken together, the results demonstrate that rNCSCs acquire a SCSC identity in the presence of EGF and FGF and that the reprogramming can be efficiently blocked by BMP4. On the other hand, NCSCs derived from adult palate rather acquire mesenchymal fates and do not acquire a CNS identity under the conditions used.

7. Zusammenfassung

Die Neuralleiste besteht aus multipotenten Neuralleisten-Stammzellen (NLSZ). Im Rumpfbereich besitzen diese Stammzellen das Potential Neurone und Glia peripherer Ganglien zu bilden (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991; Baroffio et al, 1988; Baroffio et al., 1991). Außerdem entstehen aus ihnen die dorsal gelegenen Melanozyten (Zusammengefasst in Squire, 2008). Die Neuralleistenzellen im cranialen Bereich des Körpers besitzen das zusätzliche Potential, mesenchymale Derivate, wie z.B. große Teile des Gesichtkeletts, Fettgewebe, Haut, Perizyten, sowie Bindegewebe im Nackenbereich, zu generieren (Zusammengefasst in Le Douarin, 2004).

Zellen mit NLSZ Eigenschaften wurden auch in vielen postmigratorischen Geweben, die sich von der Neuralleiste ableiten, wie z.B. im DRG (Hinterwurzelganglion), Ischiasnerv und enterischen Nervensystem, nachgewiesen (Duff et al., 1992; Morrison et al., 1999; Kruger et al, 2002).

Nach ersten Hinweisen (Svenningsen et al., 2004; Dromard et al., 2004) wurde in parallelen Studien von Baron van Evercooren (2009, 2011) und Rohrer (2011) gezeigt, dass postmigratorische NLSZ in vitro und in vivo zu ZNS-Zelltypen differenzieren können (Zujovic et al., 2009; Zujovic et al., 2011; Binder et al., 2011). Zellen aus der sogenannten Boundary Cap von E10.5 Mausembryonen können in vitro und in vivo zu Oligodendrozyten differenzieren (Zujovic et al., 2009; Zujovic et al., 2011). Die Boundary Cap entsteht aus Zellen der Neuralleiste. Sie liegt angrenzend an das Neuroepithel des Neuralrohrs. Dort münden die dorsalen sensorischen Afferenzen (dorsal root entry Zone) in das Neuroepithel und es verlassen die ventralen motorischen Efferenzen das Neuralrohr (Golding und Cohen, 1997). Die Boundary Cap enthält multipotente Stammzellen, die auf die Differenzierung von sensorischen Zelltypen spezialisiert sind (Hjerling-Leffler et al., 2005; Altman und Bayer, 1984).

Die Arbeitsgruppe Rohrer konnte zeigen, dass Sox10-positive NLSZ aus dem DRG von E12.5 Mausembryonen das Potential zur Selbsterneuerung aufweisen und Neu-

rosphären (NS) generieren (Binder et al., 2011). Nach mehreren Passagen exprimieren diese NS-Zellen den ZNS-Transkriptionsfaktor Olig2 und differenzieren zu ZNS-Neuronen, Astrozyten und Oligodendrozyten in vitro und in vivo (Binder et al., 2011). Die PNS-Marker p75 und Peripherin sind in den NS Kulturen nicht nachweisbar (Binder et al., 2011; Marlen Weber, Diplomarbeit, 2010). In vivo Experimente mit GFP-markierten Zellen zeigten, dass NS-Zellen, welche in die Gehirne von postnatalen *shiverer*-Mäusen injiziert wurden, zum größten Teil zu Zellen der Oligodendrozyten-Linie differenzieren und Axone myelinisieren. In keinem der implantierten Tiere wurden Tumore beobachtet (Binder et al., 2011). Diese Ergebnisse zeigten, dass die NLSZ zu Stammzellen mit ZNS-Eigenschaften rNLSZ (reprogrammierte Neuralleisten Stammzellen) reprogrammiert werden. Diese Befunde implizieren, dass NLSZ eine potentielle Quelle für die regenerative Medizin im ZNS darstellen. In dieser Arbeit sollte die Reprogrammierung von NLSZ aus dem DRG von E12.5 Mausembryonen zu Zellen mit ZNS-Eigenschaften im Detail untersucht werden. Außerdem wurde angestrebt NLSZ aus adultem Gewebe zu isolieren und daraus ZNS-Stammzellen zu generieren.

Die Ergebnisse dieser Arbeit zeigen, dass NLSZ aus dem DRG von E12.5 Mausembryonen in Gegenwart von EGF und FGF zu Zellen mit einer ZNS-Identität reprogrammiert werden. Die Reprogrammierung beginnt zu einem frühen Zeitpunkt, in Passage 1. Zu diesem Zeitpunkt exprimiert bereits die Mehrheit der Zellen den ZNS-Transkriptionsfaktor Olig2 und der PNS-Marker p75 wird stark herunterreguliert. Eine einheitliche Olig2 Expression und der Verlust der p75 Expression wird in NS-Zellen der Passage 3 beobachtet.

Die Daten weisen darauf hin, dass NLSZ direkt zu Zellen mit ZNS-Identität umgewandelt werden, wobei ein genetisches Programm herunterreguliert und ein anderes hochreguliert wird, ohne einem transienten Zustand bei dem zwei unterschiedliche genetische Programme aktiv sind. Ein transient pluripotenter Zustand kann ausgeschlossen werden, da in NS der 1. und 3. Passage die Expression von Pluripotenzgenen sehr schwach oder nicht feststellbar ist.

7. Zusammenfassung

Durch den Vergleich der Genexpressionsprofile von rNLSZ und Rückenmark-Stammzellen (RMSZ) konnte ermittelt werden, dass rNLSZ in Kultur eine RMSZ-Identität erhalten. Das Expressionsmuster der beiden Stammzellpopulationen ist identisch und Gene, die in der Neuralleiste und Neuralleistenderivaten exprimiert werden sind in rNLSZ und RMSZ undedektierbar. Andererseits werden Gene, die in der Ventrikulärzone und im ZNS-neuralen Epithel aktiv sind, sehr stark in rNLSZ und RMSZ exprimiert.

Die Ergebisse unterstützen die Schlussfolgerung von einer früheren Arbeit der AG Rohrer, dass rNLSZ in Kultur eine ventrale Rückenmarksidentität erhalten (Binder et al., 2011), da rNLSZ und RMSZ Gene exprimieren, die im ventralen Neuralrohr vorkommen. Andererseits sind Gene, die im Vorderhirn und Kortex exprimiert werden, nicht nachweisbar.

Die Daten von Binder et al., (2011) legten nahe, dass die ZNS-Identiät der rNLSZ stabil ist, da ins Gehirn injizierte rNLSZ keine PNS-Zellen bilden. In der vorliegenden Arbeit wurde gezeigt, dass rNLSZ auch in Kultur eine stabile ZNS-Identität besitzen. Die Zugabe von BMP4 zu den Kulturen von rNLSZ in Passage 3 konnte keine Expression von PNS-typischen Genen induzieren. Auch nach der Zugabe von BMP4 wird die Expression von Olig2 aufrechterhalten und die Zellen behalten das Potential zu ZNS-Zelltypen zu differenzieren. Zudem wird keine Schwannzelldifferenzierung induziert, wenn rNLSZ mit Faktoren behandelt werden, welche die Schwannzelldifferenzierung unterstützen.

Da rNLSZ mit einer hohen Menge an Wachstumsfaktoren, EGF und FGF, behandelt werden, liegt die Annahme nahe, dass diese Faktoren für die Reprogrammierung verantwortlich sind. Es stellte sich heraus, dass EGF nicht benötigt wird, aber FGF essentiell für die NS-Kultivierung ist. DRG-Zellen, die nur mit EGF behandelt werden bilden keine sekundären NS, während FGF-behandelte Zellen des DRGs (FGF-NLSZ) NS bilden, die mindestens bis Passage 3 kultiviert werden können. FGF-NLSZ exprimieren Olig2 und können zu Oligodendrozyten differenzieren.

Interessanterweise konnte durch die BMP4-Zugabe zu Beginn der Kultivierung die

Reprogrammierung der NLSZ (BMP NLSZ) blockiert und die PNS-Identität erhalten werden. Olig2 wird weder in Passage 1 noch in späteren Passagen von BMP NLSZ beobachtet. Dagegen bleibt die Expression von Neuralleistenmarkern, wie p75 und Sox10, in Passage 1 BMP NLSZ erhalten, wobei p75 nur in einer kleinen Subpopulation der Zellen vorkommt. Sox10 wird dagegen in 45% der Zellen exprimiert. Sox10-positive und p75-negative Zellen könnten PNS-Gliazellen darstellen.

Passage 3 BMP NLSZ können zu Neuralleistenderivaten differenzieren. In Gegenwart von Faktoren, welche die Schwannzelldifferenzierung fördern, weisen BMP NLSZ das Potential auf zu O4/p75-doppelpositiven peripheren Gliazellen zu differenzieren. Unter Proliferationsbedingungen exprimiert ein Teil der Tuj1-positiven Zellen Phox2b, ein Markergen welches in allen Neuronen und Neuronenvorläufern des autonomen Nervensystems vorkommt (Schneider et al., 1999; Reissmann et al., 1996; Shah et al., 1996). Außerdem differenzieren BMP NLSZ in der Gegenwart von Neurotrophinen zu Peripherin/Tuj1-coexprimierenden peripheren postmitotischen Neuronen.

Zusätzlich zeigen Genexpressionsprofile, dass BMP NLSZs und rNLSZs ein unterschiedliches Genexpressionsmuster aufweisen. Die Expression von ZNS-Genen in BMP NLSZ ist unterhalb der Nachweisgrenze. Andererseits exprimieren BMP NLSZ Gene, die charakteristisch für Zellen der Neuralleiste und Neuralleistenderivaten sind. Außerdem wurden Gene in BMP NLSZ detektiert, die im Mesenchym, mesenchymalen Stammzellen und Perizyten vorkommen. Im Gegensatz zu Neuralleistenzellen im Rumpfbereich, können cranial gelegene Neuralleistenzellen zu mesenchymalen Derivaten differenzieren (Zusammengefasst in Le Douarin, 2004). Die Differenzierung von Rumpf Neuralleistenzellen zu mesenchymalen Derivaten wurde bisher nur in niederen Wirbeltieren beschrieben, bei denen die dorsal gelegene Flosse von der Neuralleiste abstammt (Kague et al., 2012). In vitro wurde bereits beschrieben, dass NLSZ aus dem Rumpfbereich unter bestimmten Kulturbedingungen zu mesenchymalen Derivaten differenzieren können (John et al., 2011). Es wird vermutet, dass hierbei der TGF-β Signalweg eine wichtige Rolle spielt und das Sox10 die mesenchymale Differenzierung inhibiert (John et al., 2011). Die Daten dieser Arbeit legen nahe,

dass BMP4, ein Mitglied der TGF-β-Familie, die Differenzierung der BMP NLSZ zu mesenchymalen Zellen induzieren könnte. Außerdem wird vermutet, dass die mesenchymale Induktion in den Sox10-negativen Zellen stattfindet und Sox10-positive Zellen neurale Nachkommen der Neuralleiste darstellen.

Die Ergebnisse implizieren, dass die PNS- versus ZNS-Identität durch die antagonistische Funktion von FGF und BMP4 kontrolliert wird. Diese beiden Faktoren spielen bereits eine wichtige Rolle während der Embryonalentwicklung der Neuralleiste und dem neuralen (ZNS) Ektoderm.

Für eine angewandte Zelltherapie im ZNS muss die Quelle für die rNLSZ-Gewinnung leicht zugänglich sein und aus dem adulten Organismus stammen. Im Palatum von adulten Ratten wurden Nestin/S100/p75-positive NLSZ in der Innervation von Meissner Korpuskeln nachgewiesen. Es wird vermutet, dass diese Zellen das Potential aufweisen, NS zu generieren (Widera et al., 2009; Widera et al., 2011). In der vorliegenden Arbeit sollte ermittelt werden, ob NLSZ aus dem Palatum von adulten Mäusen (pNLSZ) in Gegenwart von EFG und FGF das Potential besitzen, ZNS-Zelltypen zu generieren. Es wurde zunächst gezeigt, dass die NS-bildenden Zellen von Sox10-positiven NLSZ aus dem Gewebe abstammen. Während der NS-Kultivierung werden die Neuralleistenmarker, p75 und Sox10, sehr stark herunterreguliert. Eine Induktion von ZNS-Genen konnte jedoch nicht beobachtet werden. Der Vergleich der Genexpressionsprofile zeigten, dass pNLSZ und rNLSZ ein unterschiedliches Genexpressionsmuster aufweisen. pNLSZ exprimieren Gene sehr stark, welche in der Neuralleiste und vor allem in mesenchymalen Neuralleistenderivaten, mesenchymalen Stammzellen und Perizyten vorkommen. Perizyten und der größte Teil des Mesenchyms des Palatums stammen von der cranialen Neuralleiste ab (Ito et al., 2003; Zusammengefasst in Le Douarin, 2004). Dies deutet darauf hin, dass pNLSZ in NS Kulturen präferentiell zu mesenchymalen Stammzellen/Perizyten werden, also ein normales Entwickungspotential der cranialen Neuralleiste verwirklichen. Es ist unklar weshalb pNLSZ nicht zu ZNS-Stammzellen reprogrammiert werden. Da jedoch in mehreren Arbeiten gezeigt wurde, dass das Entwicklungspotential von NLSZ

mit zunehmenden Alter eingeschränkt wird, ist anzunehmen, dass dies auch den Unterschied zwischen rNLSZ und pNLSZ ausmacht (Zusammengefasst in Delfino-Machìn et al., 2007).

Zusammenfassend kann festgestellt werden, dass NLSZ aus dem embryonalen DRG in Gegenwart von EGF und FGF eine ZNS-Identität durch direkte Reprogrammierung erlangen und BMP4 die Reprogrammierung blockiert. Eine Reprogrammierung von adulten pNLSZ des Palatums zu Zellen mit ZNS-Identität wird unter den Kultivierungs-bedingungen nicht beobachtet.

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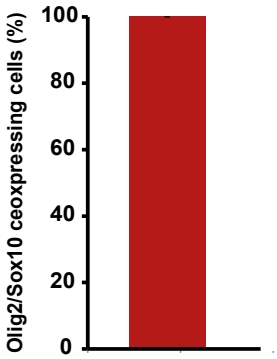
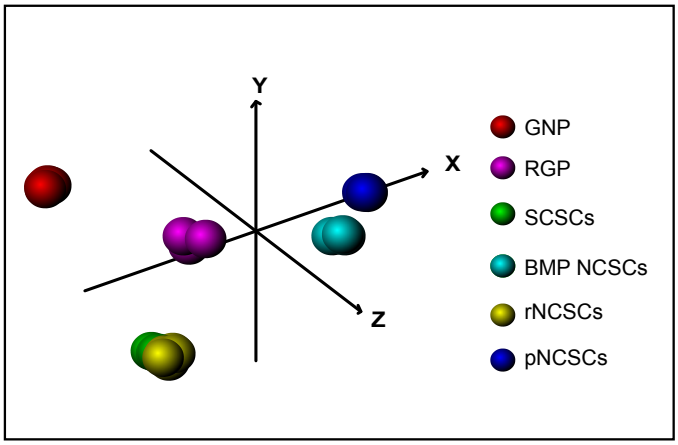
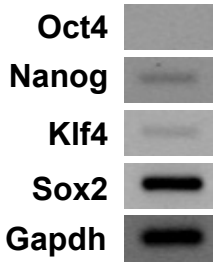
9. Supplementary Figures



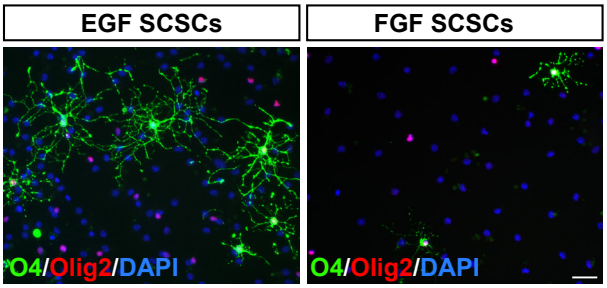
Supplementary Figure 1: Olig2 is coexpressed in virtually all Sox10-positive cells of rNCSCs. Quantifications of immunostainings of 3rd passage rNCSCs indicate that virtually all Sox10-positive cells coexpress Olig2. n=3; mean ± SEM.



Supplementary Figure 2: PCA (principal component analysis) of expression profiles of rNCSCs, SCSCs, BMP NCSCs, pNCSCs, GNPs (granule neuron progenitors) and RGPs (radial glia progenitors). Unsupervised PCA comparing 3 replicates of each cultured 3rd passage rNCSCs, SCSCs, BMP NCSCs, pNCSC, RGPs and GNPs. Each dot represents the global gene expression of a single microarray sample. Distances between dots indicate the difference between the samples. Weber et al., 2015.

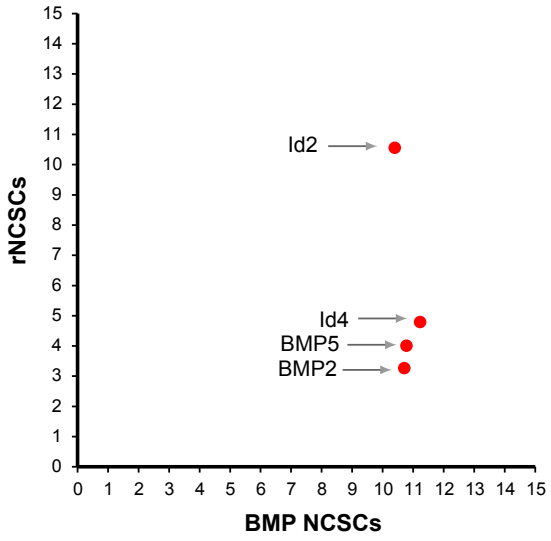


Supplementary Figure 3: Passage 1 rNCSCs express pluripotency genes at a low level. RT-PCRs of 1. passage rNCSCs reveal high expression of Sox2. Klf4 and Nanog a expressed at low levels and Oct4 is undetectable.

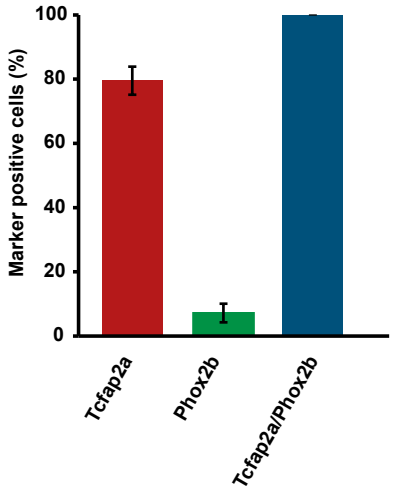


Supplementary Figure 4: 3rd passage SCSCs either cultured with EGF (EGF SCSCs) or FGF (FGF SCSCs) generate oligodendrocyte progeny. 3rd passage EGF SCSCs and FGF SCSCs differentiate into O4/Olig2-co-expressing oligodendrocyte-like cells, analyzed via immunocytochemistry. Scale bar: 30 µm

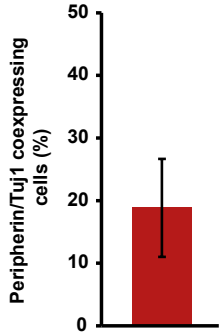
9. Supplementary Figures



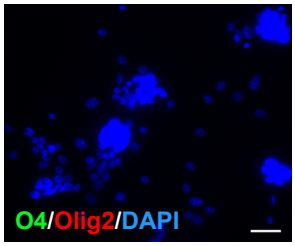
Supplementary Figure 5: BMPs and BMP down stream target genes are expresssed at high levels in BMP NCSCs. Scatter plot of a small subset of genes from microarray data demonstrates that BMP NCSCs express BMP2, BMP5, Id2 and Id4 at high levels. rNCSCs only express Id2 at high levels, wheras Id4, BMP2 and BMP5 are expressed at background or undetectable levels.



Supplementary Figure 6: BMP NCSCs contain Tcfap2a and Phox2b positive cells under proliferation conditions. Quantifications of immunostainings of 3rd passage BMP NCSCs reveal expression of Tcfap2a (79,5% ± 4,4%) and Phox2b-positive cells (7,3% ± 3%). 100% of Phox2b-positive cells coexpress Tcfap2a. n=3; mean ± SEM.



Supplementary Figure 7: BMP NCSCs differentiate into Peripherin/Tuj1 coexpressing neurons. Quantification of immunostainings reveal that 18,8% ± 7,8% of Tuj1-positive neurons coexpress Peripherin after the differentiation in the presence of neurotrophins. n=3; mean ± SEM.



Supplementary Figure 8: After delayed BMP4 removal, BMP NCSCs lack CNS marker genes. Immunostainings of passage 5 BMP NCSCs after delayed BMP4 removal indicate no upregulation of Olig2 or O4. This was analyzed under proliferation conditions on short term cultures. Scale bar: 30 µm.

Versicherung

Ich erkläre hiermit, dass ich die vorgelegte Dissertation über

„**Reprogramming of stem cells in the peripheral nervous system to CNS stem cells“**

selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.
Ich versichere, die Grundsätze der guten wissenschaftlichen Praxis beachtet, und nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

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