Heinrich-Heine-Universität Düsseldorf

Fate modulation of oligodendroglial progenitor cells and adult neural stem cells

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"There are more things in heaven and earth, Horatio,
Than are dreamt of in your philosophy."
Shakespeare, Hamlet (I.5.167), 1564-1616

„Alle sagten: Das geht nicht. Dann kam einer,
der wusste das nicht und hat es gemacht!"
Unbekannt

To dla Cebie, Babciu!

HEINRICH-HEINE-UNIVERSITÄT DÜSSELDORF

Abstract

Mathematisch-Naturwissenschaftliche Fakultät
Department of Neurology

Doctor rerum naturalium

Fate modulation of oligodendroglial progenitor cells and adult neural stem cells

by Janusz Joachim Jadasz

Myelin loss occurs in the event of damage to the central nervous system (CNS) and in diseases such as multiple sclerosis. This may lead to axonal degeneration concomitantly resulting in visual and motor deficits. The development of meaningful repair strategies for the demyelinated CNS is currently being investigated. The question of how to stimulate the genesis and maturation of myelin-forming cells is an important part of that investigation. These so-called oligodendrocytes arise from resident oligodendroglial progenitor cells and from adult neural stem cells. The scientific challenge is to identify factors and mechanisms determining oligodendroglial cell fate and promoting differentiation into mature oligodendrocytes. In this context the intrinsic regulation of $p57^{Kip2}$ in the peripheral nervous system has been described as leading to the differentiation of mature myelin-building Schwann cells. Moreover, it was shown that mesenchymal stem cells secrete as yet unknown factors which ameliorate oligodendroglial differentiation of adult neural stem cells in the CNS. Based on these observations, the present study examines whether $p57^{Kip2}$ modulates glial fate decision of adult neural stem cells and whether $p57^{Kip2}$ regulates oligodendroglial progenitor cell differentiation in the CNS. Furthermore, this work aims to address the question of whether the extrinsic application of mesenchymal secreted factors modulates oligodendroglial fate of progenitor cells, which additionally have the potential to develop into astrocytes, and whether these factors are also involved in the final maturation. The first approach was to intrinsically modulate progenitor cells and adult neural stem cells using shRNA-mediated down-regulation of $p57^{Kip2}$ mRNA. In the second, oligodendroglial progenitor cells were extrinsically incubated in a medium conditioned with mesenchymal stem cells. Immunocytochemical stainings and gene expression analysis were applied at different time points for qualitative and quantitative study of the glial cells. The results show that the suppression of $p57^{Kip2}$ leads to oligodendroglial fate decision in adult neural stem cells and ameliorates oligodendroglial progenitor cell differentiation. Interestingly, transplantation of

$p57^{Kip2}$-suppressed adult neural stem cells into the spinal cord results in oligodendroglial marker expression even under the astrocytic conditions prevailing there. Furthermore, mesenchymal factors stabilize the oligodendroglial fate of progenitor cells and accelerate their maturation. Both intrinsic and extrinsic modulation repress astrocytic development of the multipotent cells. In conclusion, it was found that the cell fate of oligodendroglial progenitor cells and adult neural stem cells can be modulated by both intrinsic and extrinsic regulation. This takes place on an oligodendroglial-astroglial modulation axis. The modulation mechanisms proposed here provide a basis for the development of future cell replacement strategies aiming at remyelination.

Zusammenfassung

Bei Verletzungen im zentralen Nervensystem (ZNS) und bei Erkrankungen wie der Multiplen Sklerose kann es zum Verlust der Myelinscheiden an Axonen kommen. Dies kann zu axonaler Degeneration führen und es treten visuelle und motorische Defizite auf. Um dem entgegen zu wirken, wird untersucht wie man die Genese und Reifung myelinbildender Zellen stimulieren kann. Diese als Oligodendrozyten bezeichneten Zellen können einerseits aus residenten oligodendroglialen Vorläuferzellen heranreifen oder aus adulten neuralen Stammzellen hervorgehen. Die Herausforderung besteht in der Identifizierung von Faktoren und Mechanismen, die zum einen das Zellschicksal zur oligodendroglialen Zellidentität bestimmen und zum anderen die Ausdifferenzierung zu reifen Oligodendrozyten fördern. Im peripheren Nervensystem konnte in diesem Zusammenhang beschrieben werden, dass die intrinsische Regulation des $p57^{Kip2}$ Gens zur Differenzierung von reifen myelinbildenden Zellen führt. Darüberhinaus wurde gezeigt, dass mesenchymale Stammzellen noch nicht beschriebene Faktoren sezernieren, welche neurale Stammzellen im ZNS zu reifen oligodendroglialen Zellen auswachsen lassen. Basierend auf diesen Beobachtungen soll in der vorliegenden Arbeit untersucht werden, ob $p57^{Kip2}$ einen Regulator der Differenzierung oligodendroglialer Vorläuferzellen sowie einen glialen Schicksalsdeterminanten adulter neuraler Stammzellen im ZNS darstellt. Zudem soll gezeigt werden, inwieweit oligodendrogliale Vorläuferzellen, die außerdem das Potenzial besitzen zu Astrozyten auszuwachsen, über mesenchymale Faktoren in ihrem Zellschicksal determiniert werden und ob diese Faktoren ebenfalls an der endgültigen Ausreifung beteiligt sind. In einem intrinsischen Ansatz wurden zunächst oligodendrogliale Vorläuferzellen und adulte neuralen Stammzellen mittels einer shRNA-vermittelten Suppression der $p57^{Kip2}$ mRNA moduliert und im Hinblick auf ihre Differenzierung und Zellgenerierung untersucht. In einem weiteren extrinsischen Ansatz wurden oligodendrogliale Vorläuferzellen mit einem durch mesenchymale Stammzellen konditionierten Medium inkubiert. Zur qualitativen und quantitativen Erfassung der glialen Eigenschaften der Zellen wurden immunzytochemische Färbungen und Genexpressionsanalysen an verschiedenen Zeitpunkten angewandt. Es konnte gezeigt werden, dass die Suppression von $p57^{Kip2}$ die Differenzierung oligodendroglialer Vorläuferzellen beschleunigt. Darüberhinaus führt diese Modulation in adulten neuralen Stammzellen zu einer Schicksalsdeterminierung in Richtung oligodendroglialer Zellidentität. Auch konnte nachgewiesen werden, dass $p57^{Kip2}$-supprimierte adulte neurale Stammzellen nach Transplantation in das Rückenmark, trotz des dort herrschenden astrozytären Milieus, oligodendrogliale Marker aufweisen. Weiterhin konnten wir nachweisen, dass die extrinsische Zugabe sezernierter mesenchymaler Faktoren bei oligodendroglialen Vorläuferzellen eine finale Schicksalsentscheidung in Richtung oligodendroglialer Achse festigt. Des Weiteren führt diese Zugabe zu einer schnelleren Ausreifung dieser Zellen. Sowohl bei der intrinsischen,

als auch bei der extrinsischen Modulation werden astrozytäre Charakteristika durch $p57^{Kip2}$ oder mesenchymale Faktoren unterdrückt. Zusammenfassend lässt sich herausstellen, dass wir sowohl über die intrinsische als auch über die extrinsische Regulation das Zellschicksal adulter neuraler Stammzellen und oligodendroglialer Vorläuferzellen modulieren können. Dies geschieht entlang einer oligodendroglialen-astroglialen Modulationsachse. Die hier vorgestellten Modulationsmechanismen stellen die Grundlage für zellersatzbasierende Therapieansätze im Tiermodel dar.

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Abbreviations

aNSC	**a**dult **N**eural **S**tem **C**ell
Ascl1	**A**chaete-**s**cute **c**omplex **l**ike **1**
AQP4	**AQ**ua**P**orin-**4**
BBB	**B**lood-**B**rain **B**arrier
BDNF	**B**rain **D**erived **N**eurotrophic **F**actor
BWS	**B**eckwith-**W**iedemann **S**yndrome
bFGF	**b**asic **F**ibroblast **G**rowth **F**actor
bHLH	**b**asic **H**elix-**L**oop-**H**elix
BMP	**B**one **M**orphogenetic **P**rotein
CA	**C**ornu **A**mmonis
CDK	**C**yclin-**D**ependent **K**inase
CDKI	**C**yclin-**D**ependent **K**inase **I**nhibitor
CDKN1C	**C**yclin-**D**ependent **K**inase i**N**hibitor **1C**
Cip1	**C**DK-**i**nteracting **p**rotein **1**
CNPase	2',3'-**C**yclic **N**ucleotide 3'- **P**hophodieste**rase**
CNS	**C**entral **N**ervous **S**ystem
CNTF	**C**iliary **N**euro**T**rophic **F**actor
DCX	**D**ouble**C**ortin on **X** chromosome
DG	**D**entate **G**yrus
E10-E18	**E**mbryonic day **10-18**
EAE	**E**xperimental **A**utoimmune **E**ncephalomyelitis
EGF	**E**pidermal **G**rowth **F**actor
ESC	**E**mbryonic **S**tem **C**ell
FBS	**F**etal **B**ovine **S**erum
FGF-2	**F**ibroblast **G**rowth **F**actor**-2**

GalC	**Gal**acto**C**erebroside
GC	**G**ranular **C**ell
GCL	**G**ranular **C**ell **L**ayer
Gcm	**G**lial **c**ells **m**issing
GFAP	**G**lial **F**ibrillary **A**cidic **P**rotein
GLAST	**G**lutamate **T**ransporter **P**rotein
GM	**G**rey **M**atter
G-N	**G**lial-**N**euronal axis
HC	**H**ippo**C**ampus
Hes	**H**airy/**e**nhancer of **s**plit
HGF	**H**epatocyte **G**rowth **F**actor
Id	**I**nhibitor of **d**ifferentiation
IL-6	**I**nter**L**eukin-**6**
iPSC	**i**nduced **P**luripotent **S**tem **C**ell
Kip2	**K**inase **i**nhibitory **p**rotein **2**
LIF	**L**eukemia **I**nhibitory **F**actor
LIMK-1	**LIM K**inase **1**
LV	**L**ateral **V**entricle
MAG	**M**yelin-**A**ssociated **G**lycoprotein
Mash1	**M**ammalian **a**chaete-**s**cute complex **h**omolog like **1**
MBP	**M**yelin **B**asic **P**rotein
ML	**M**olecular **L**ayer
MOG	**M**yelin **O**ligodendrocyte **G**lycoprotein
MS	**M**ultiple **S**clerosis
MSC	**M**esenchymal **S**tem **C**ell
MSC-CM	**M**esenchymal **S**tem **C**ell-**C**onditioned **M**edium
NE	**N**euro**E**pithelium
NeuN	**Neu**ron-**s**pecific **N**uclear protein
NG2	**N**euron-**G**lial antigen **2**
Ngn	**N**euro**gen**in
NT3	**N**euro**T**rophin **3**
OKMS	**O**ct4, **K**lf4, c-**M**yc, **S**ox2
O-2A	**O**ligodendrocyte-type**2**-**A**strocyte

O-A	**O**ligodendroglial-**A**stroglial axis
OB	**O**lfactory **B**ulb
OPC	**O**ligodendroglial **P**rogenitor/precursor **C**ell
OL	**OL**igodendrocyte
P0	myelin **P**rotein **0**
PC	**P**yramidal **C**ell
PCNA	**P**roliferating **C**ell **N**uclear **A**ntigen
PDGF	**P**latelet **D**erived **G**rowth **F**actor
PDGFR-α	**P**latelet **D**erived **G**rowth **F**actor **R**eceptor-α
PLP	**P**roteo **L**ipid **P**rotein
PMP22	**P**eripheral **M**yelin **P**rotein **22**
PNS	**P**eripheral **N**ervous **S**ystem
PSA-NCAM	**P**oly**S**ialic **A**cid-**N**eural **C**ell **A**dhesion **M**olecule
RA	**R**etinoic **A**cid
RG	**R**adial **G**lia cell
RMS	**R**ostral **M**igratory **S**tream
SCI	**S**pinal **C**ord **I**njury
SDF-1	**S**tromal **D**erived **F**actor-**1**
SGZ	**S**ub **G**ranular **Z**one
Sox	**S**ex determining region Y b**ox**
SVZ	**S**ub **V**entricular **Z**one
TF	**T**ranscription **F**actor
TGF	**T**ransforming **G**rowth **F**actor
TH	**T**yrosine **H**ydroxylase
Th1-cells	Type **1** **h**elper **T**-**cells**
VZ	**V**entricular **Z**one
Waf1	**W**ild-type p53-**a**ctivated **f**ragment **1**
WM	**W**hite **M**atter
USSC	**U**nrestricted **S**omatic **S**tem **C**ell
ZNS	**Z**entrales **N**erven **S**ystem

Chapter 1

Introduction

All higher life forms are controlled by their nervous system. Conscious as well as subconscious commands regulate the highly complex interchange of information. Specialized cells fulfil this finely tuned task in the central nervous system (CNS). Other specialized cells are responsible for the maintenance of cell homeostasis as well as potential recovery of damaged tissue after injury to the CNS. This broad cell variety is based on progenitor and stem cells in the CNS, which give rise to various neural cell lineages. However, the regenerative capacity of the adult brain is limited. Modulation of fate decision in adult multipotent cell types therefore represents a promising task of neuroscience research in enhancing the regenerative capacity of the CNS.

Our aim is to study novel fate regulators of oligodendroglial progenitor cells (OPCs) and adult neural stem cells (aNSCs) that modulate cell differentiation. This thesis describes cell fate modulation of OPCs and aNSCs by intrinsic or extrinsic factors: regulation of $p57^{Kip2}$ in intrinsic modulation and modulation via extrinsic mesenchymal stem cell secreted factors.

1.1 The (central) nervous system — different cell types and their function

The mammalian nervous system processes incoming as well as outgoing stimuli in order

to manage the complex exchange of information. The main tasks of the peripheral nervous system (PNS) are forwarding organ and tissue-specific stimuli (afferent or sensory signals) to the spinal cord and brain. The CNS processes the forwarded PNS stimuli and transmits efferent or motoric signals to the periphery. A signalling route in the nervous system thus comprises a minimum of two different conduction pathways built by neurons. A typical mammalian neuron is phenotypically characterized by its cell body or perikaryon containing the nucleus. The neuron possesses multiple numbers of cellular extensions called dendrites and usually one axon, which may span the hundredfold length of the cell body within the CNS or to the periphery. The dendrites and soma act mostly as receivers of upstream neuronal signals via special structures called synapses, which are constructed by the axons of neighbouring neurons. To this end, the axon terminal of one neuron and the dendrite of the other communicate via the synaptic release of neurotransmitters. Neurotransmitters bind to receptors located on the dendritic or somatic post-synapses to initiate downstream signal transmission. This downstream signalling leads in the axon hillock to action potential generation, characterized as the active depolarization of the membrane. The depolarization takes place along the axon either to the next synapse or to peripheral targets such as muscles.

Apart from the neuronal cells of the nervous system another main class of cells is known — the glial cells. The term glia covers different cell types in the CNS and the PNS such as Schwann cells (in the PNS), astrocytes, microglia, ependymal cells, pericytes, radial glial cells (RGs) and oligodendrocytes (OLs), together with their progenitors (all in the CNS) [reviewed in Pfrieger and Slezak, 2012]. All these different cell types are known today for their diverse functions in the organization of the nervous system. For instance, microglia are the phagocytes of the CNS and are activated in response to damage or infection [reviewed in Czeh et al., 2011]. Ependymal cells control the circulation of cerebrospinal fluid and line the fluid-filled cavities of the CNS [reviewed in Bruni, 1998]. Astrocytes play key roles in neuronal activity by regulating ionic homeostasis in the synaptic extracellular space, metabolic support of neurons with nutrients, synaptogenesis and neurotransmitter release [reviewed in Costa et al., 2010]. In addition, astrocytes, ependymal cells and pericytes separate the brain from the rest of the body by the blood-brain barrier (BBB) [reviewed in Wolburg et al., 2009]. The expression of glial fibrillary acidic protein (GFAP) is characteristic of astrocytes [reviewed in Götz and Huttner, 2005]. Their name originates from the Greek for star in reference to their star-like cell shape. Another noteworthy GFAP-positive cell subtype is the so-called

radial glial cell. During development, RGs are known to guide neurons in their migration through the neocortex [Rakic, 1971] and also have the capacity to differentiate into neuronal progenitors [Noctor et al., 2001]. Moreover, oligodendrocytes wrap axons in a lipid layer known as myelin that protects the axon and enhances the velocity of signal transmission. The equivalent of axon-ensheathing cells in the PNS are the Schwann cells. One major difference between these cells of similar function is that Schwann cells coat a single axon, while oligodendrocytes are able to produce the lipid layer for up to 50 different axons.

The adult CNS of vertebrates can be morphologically classified into grey matter (GM, *Substantia grisea*) and white matter (WM, *Substantia alba*). WM consists of axons and glial cells, and its peculiar white colour is attributed to the high content of myelin. Cell bodies of neuronal cells are resident in the GM. In contrast to WM, these areas appear grey due to a low concentration of myelin.

1.2 The development of the central nervous system

The formation of the neural tube is the starting point for the development of the functional CNS including spinal cord and brain. Glial cells and neurons originate from a single cell-layer composed of embryonic neural stem cells, the so-called neuroepithelium (NE), which lines the cerebral ventricle, the spinal canal and the optic canal in the embryonic stage. These neuroepithelial cells increase in number prior to their asymmetric cell division and are mainly responsible for tangential growth of brain regions, which is then followed by radial growth [Rakic, 1995]. During development, the NE becomes a multilayer structure forming its inner ventricular zone (VZ) — the primary proliferative zone. Originating from the NE within the VZ, neurons, astrocytes and oligodendrocytes evolve in chronological sequences starting with the development of neurons (neurogenesis). Neuronal development can be observed between embryonic day 10 (E10) and E17 in mouse CNS, whereas astrocyte generation begins on E16 (starting point for gliogenesis) and peaks postnatally during the first week. By contrast, mature oligodendrocytes emerge at postnatal stages only.

1.2.1 Neurogenesis

The differentiation of stem cells into neurons is divided into different phases: proliferation, differentiation, migration, axonal and dendritic generation and synaptogenesis. Transcription factors (TFs) with basic helix-loop-helix (bHLH) motifs are among the main drivers of the complex network of stages in the development of neural stem cells in the embryonic brain. At the beginning of neurogenesis, cell division of the neural stem cells changes from symmetric to asymmetric (E10-E13 in mouse development) [reviewed in Götz and Huttner, 2005]. Asymmetric cell division, however, causes one stem cell to remain in the epithelium and a daughter cell to migrate along radial glial fibres into the cortex to mature into a neuronal cell. The number of neural stem cells thus remains constant even though the number of neurons is growing. Hairy/enhancer of split (Hes) and inhibitor of differentiation (Id) inactivation characterize the next phase of differentiation, in which proneuronal bHLH TFs neurogenin (Ngn) and mammalian achaete-scute complex homolog 1 (Mash1 or Ascl1) are activated [reviewed in Corbin et al., 2008]. Each step in differentiation can be identified by the expression of distinct marker proteins within neural stem cells, neuronal progenitor cells and mature cell types. For instance, transition of a stem cell into a neuron is accompanied by the loss of nestin expression in the neuronal progeny. By contrast, doublecortin (DCX), neuron-specific nuclear protein (NeuN) and polysialic acid-neural cell adhesion molecule (PSA-NCAM) are expressed in neuronal progenitors [Scott et al., 2010]. These neuronal progenitor cells migrate away from the proliferation zone to establish a neuronal circuit. Additionally, in the E13-E17 period, neuronal progenitors migrate radially from the VZ within the dorsal telencephalon towards the pial surface, where they build six cortical layers in an inside-out pattern in the mouse brain [reviewed in Kriegstein et al., 2006]. During the last phase, synapses are formed to build a signalling network while astrogliogenesis begins.

1.2.2 Astrogliogenesis

As the peak of neurogenesis decreases around day 16 of mouse embryonic development, gliogenesis starts with the generation of astrocytes. Radial glial cells are an exception to the chronological arrangement in the developing brain. These glial cells emerge from

neuroepithelial cells out of the VZ as neurogenesis begins and guide newborn neuroblasts towards their spatial destiny [reviewed in Kriegstein and Götz, 2003]. Interestingly, RGs also give rise to neuroblasts, which then migrate along their "mother" radial fibre guides toward the neocortex [Noctor et al., 2001]. Later, in the postnatal brain, these RGs reside in the subventricular zone (SVZ), become astrocytes [Schmechel and Rakic, 1979] and form the subventricular stem cell niche [reviewed in Tramontin et al., 2003].
However, the origin of the astrocyte progenitor can be located in the dorsal regions of the neural tube in the developing brain. Here, the inhibition of proneuronal factors acts as a driver for astroglial generation. While in neurogenesis Ngn primarily promoted neuron differentiation, this protein later indirectly regulates astrocyte birth. More specifically, after removal of Ngn, bone morphogenetic proteins (BMPs) bind to BMP-receptors and activate Smad proteins by phosphorylation. Furthermore, BMPs induce expression of Hes, which in turn leads to Ngn and Mash1 repression [Nakashima et al., 2001]. Additionally, Hes is able to activate the Notch signalling pathway — a known promoter of gliogenesis [Ge et al., 2002]. Growth and transcription factors play organizational roles in astrocytic development. Interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are the main factors currently described as exerting a positive influence on astrogliogenesis [Bonaguidi et al., 2005; Bonni et al., 1997]. When astrocytes differentiate, they also develop different morphologies according to their function in the brain. In the adult rodent, two subpopulations can be distinguished: protoplasmatic astrocytes in the GM and fibrillary astrocytes in the WM of the brain. The common feature of all astrocytes is the development of end-feet that are, for instance, wrapped around blood vessels to form the BBB [Abbott, 2002]. Furthermore, one of the first identified specific components of astrocytes is GFAP [Eng et al., 1971]. Other specific antigens such as the glutamate transporter protein (GLAST) [Shibata et al., 1997], brain lipid binding protein (BLBP) [Feng et al., 1994] or aquaporin-4 (AQP4) [Nielsen et al., 1997] are also used in the identification of astrocytes.
In the neuro- and gliogenic development sequence, oligodendroglial progenitors are formed last.

1.2.3 Oligodendrogenesis and oligodendroglial progenitor cells

Myelin-producing oligodendrocytes arise from proliferating progenitor cells (originating in the ventral telencephalon), which in turn migrate from the ventricle germ zone into

the subsequently formed WM [Small et al., 1987]. This migration occurs in the late embryonic stage as well as shortly after birth and is not fully understood, although it is known that cell adhesion molecules and chemokine gradients play a guiding role. For a functional specification of progenitor cells and stem cells towards oligodendrocytes, a controlled upregulation of specific factors is essential. The main stimuli driving the OPC differentiation are bHLH TFs such as Olig1 and Olig2 [Mei et al., 2013; Zhou and Anderson, 2002]. They promote generation of OPCs expressing the chondroitin sulphate proteoglycan neuron-glial antigen 2 (NG2). These progenitors can in turn develop into myelinating OLs [Ligon et al., 2006]. In addition, Olig2 is present in OPCs and OLs throughout life and is therefore recommended as a typical OL marker. Furthermore, Hes5 inhibits oligodendrogenesis by repressing myelin genes, also following the BMP signalling cascade. Sox10 is also important for functional development of mature oligodendrocytes by transcriptionally regulating platelet derived growth factor (PDGF)-α [Finzsch et al., 2008].

OPCs divide in cell culture on addition of PDGF and basic fibroblast growth factor (bFGF). For instance, PDGF was described as being responsible for the production of OPCs *in vivo* [Calver et al., 1998]. Furthermore, bFGF contributes to the differentiation of OPCs into mature oligodendrocytes [Collarini et al., 1991]. Under certain circumstances these cells also have the ability to turn into astrocytes. It has been shown that oligodendroglial progenitor cells can give rise to either mature oligodendrocytes (in serum-free medium) or to type-2 astrocytes (when foetal bovine serum was added to the medium). These progenitor cells display a bipolar morphology expressing A2B5, vimentin or nestin and are called oligodendrocyte-type2-astrocyte (O-2A) progenitors [Raff et al., 1983]. The O-2A cells are isolated from different regions in the postnatal CNS. However, it is not known whether O-2A progenitors are able to use their potency to differentiate into both cell types *in vivo*.

The development of an early oligodendroglial progenitor cell towards mature oligodendrocytes is characterized by the gradual expression of cell surface antigens (Fig. 1.1). For instance, proliferating and migrating bipolar progenitors express the ganglioside A2B5 and are stimulated by PDGF and bFGF. In studies to elucidate the origin of oligodendrocytes, the α receptor of PDGF (PDGFR-α) was found to be a specific marker for oligodendroglial progenitors [Hall et al., 1996]. These O-2A progenitors also express NG2 and are found around E17 in the CNS [Nishiyama et al., 1996]. In the next step in differentiation, they start to express O4-antigen while their morphology becomes more

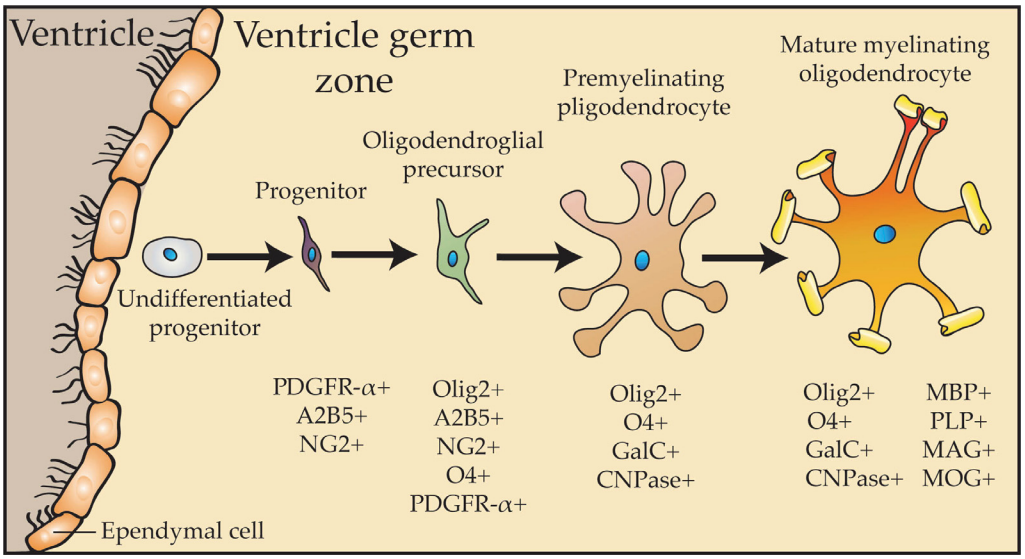

FIGURE 1.1: *Oligodendroglial differentiation of progenitor cells.* Undifferentiated progenitor cells migrate out of the ventricle zone and populate the brain. These cells differentiate from A2B5/PDGFR-α/NG2-positive progenitors into mature myelinating oligodendrocytes. Different markers are expressed at different stages and the oligodendroglial morphologies are becoming more complex.

complex with a higher number of cell processes in the premyelinating oligodendroglial progenitor stage. A2B5, PDGFR-α as well as NG2 expression disappear in the process of differentiation into premyelinating oligodendrocytes. At this stage PDGF no longer has any effect, although these cells can still proliferate on stimulation with bFGF. In the following maturation stages, OPCs begin expressing galactocerebroside (GalC) and initiate the production of mature oligodendrocyte markers such as 2', 3'-cyclic nucleotide 3'-phophodiesterase (CNPase) and proteolipid protein (PLP). In this mature oligodendrocyte stage, these cells also express myelin basic protein (MBP), myelin associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) and display a complex morphology characterized by sheath-building cell processes [reviewed in Baumann and Pham-Dinh, 2001].

1.3 Adult neural stem cells

During embryonic development of the CNS and especially while neurogenesis is in process, precursor cells settle in specific forming regions of the brain and persist to postnatal stages. After the discovery of these adult neural stem cells within the adult brain in the early 1960s [Altman and Das, 1965], it is widely accepted today that two main areas of

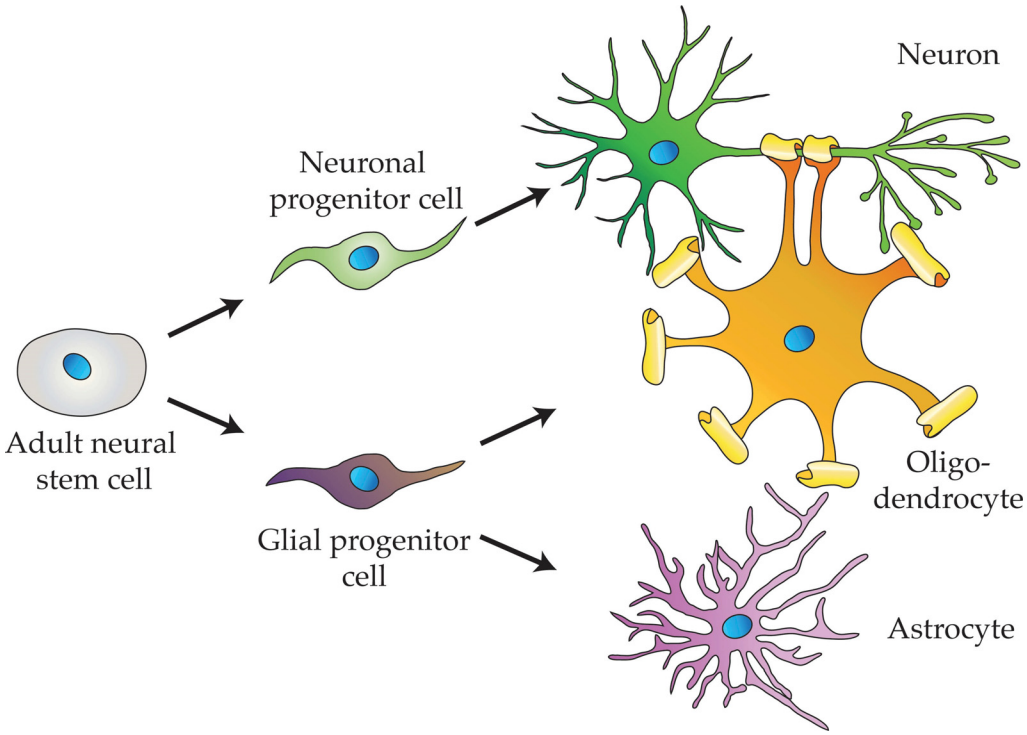

FIGURE 1.2: *Schematic illustration of fate decision of adult neural stem cells.* New born adult neural stem/progenitor cells are capable of differentiating into the three neural cell types.

the brain contain stem cell niches — the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (HC) and the SVZ along the lateral ventricle (LV). Characteristically, aNSCs are able to self-renew and can differentiate into various mature brain cell lineages such as neurons, oligodendrocytes and astrocytes (illustrated in Fig. 1.2). These phenomena can be reproduced *in vitro* in the so-called neurosphere assay. For this purpose, hippocampal and SVZ-derived tissue is prepared and cells are isolated. Culturing these cells in medium containing fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) increases the number of stem and progenitor cells, which then form cellular spheres arising out of one stem cell — the so-called neurosphere [Reynolds and Weiss, 1992]. Cells formed in neurospheres express the stem cell characteristic marker nestin [Sirko et al., 2007] and start to differentiate into the three neuronal cell lineages on removal of growth factors *in vitro* [Weiss et al., 1996].

1.3.1 The stem cell niche in the hippocampal formation

The term “hippocampus” was chosen for the area that is part of the telencephalon of

the brain and appears to be morphologically similar to a sea horse. The hippocampal formation is an important component of the limbic system and has been shown to be neurogenetically active throughout life. It is located along the lateral ventricular horn at the temporal lobe. The hippocampus is composed of the DG, the cornu ammonis (CA) and the subiculum. Three different layers characterize the DG histologically — stratum moleculare, stratum granulosum and the hilus, which is often referred to as CA4 (see Fig. 1.3). These layers contain granule cells (GCs), which are characterized as multipolar excitatory neurons with short axons and an extensive dendritic tree [reviewed in Morrens et al., 2012]. GCs are important for memory formation and learning, as they process cortical signals to the hippocampus. Furthermore, the hippocampus features the neuronal union of different cornu ammonis areas with a chain of connections between them. The area of the cornu ammonis is subdivided into the CA1 area, which consists mainly of small pyramidal cells (PCs), followed by the CA2 area of large PCs, and the CA3 area, where the PCs are not as densely packed as in CA1 or CA2 [reviewed in Jones and McHugh, 2011]. The illustration in Fig. 1.3 shows the hippocampal formation.
Proliferating cells can be observed along the boundary between the SGZ and the hilus. These cells generate a continuous pool of differentiating neuronal precursors, which in turn become integrated in the hippocampal granular cell layer (GCL). Seri and colleagues were able to demonstrate that the SGZ is thought to generate neuronal and glial progenitor cell types. Here, special progenitor stages are described: stem cells start to divide and become D1 cells, which are "transiently amplifying precursor cells". These cells pass through various intermediate steps before turning into post-mitotic granular cells, which are integrated in the neuronal network of the hippocampus [Seri et al., 2004]. Newly generated granular cells functionally establish connections in the SGZ tissue and therefore participate in signal transmission in the dentate gyrus of animals [van Praag et al., 2002]. Moreover, hippocampal aNSCs have the potential to turn into oligodendrocytes, for instance, when required [Jessberger et al., 2008].
However, the molecular mechanisms regulating the hippocampal network are still not well known.

1.3.2 The stem cell niche in the subventricular zone

In the adult brain, the architecture of the SVZ differs from the SGZ niche, as its cells are mainly subdivided into four subtypes. Fig. 1.3 shows type A class cells, which are

PSA-NCAM and DCX positive neuroblasts and the most common. These cells migrate along the rostral migratory stream (RMS) towards the olfactory bulb (OB) to form interneurons [reviewed in Yao et al., 2012]. A network of mainly astrocyte characteristic type B cells build a pathway for this purpose, and the migration process itself appears to follow a chain, which is known as chain migration. Although it is clear that humans possess a similar migratory system towards the OB [Curtis et al., 2007] as that shown in rodents, it is not clear whether this path shares a similar chain migration. Nevertheless, type B astrocytic cells are subdivided into two groups — B1 and B2 cells. B1 cells are mainly located near the epithelium, whereas B2 cells are found near the striatum [Doetsch et al., 1997]. Additionally, B cells have been shown to be stem cells in the SVZ [Doetsch et al., 1997]. Type C cells, representing the third subtype, proliferate fast and fill up cellular gaps in the type A and type B network. Further, ciliary ependymal cells (type E) make up the cellular border of the SVZ and the ventricle. It has been shown that type B cells are capable of differentiating into transit-amplifying type C cells and that these cells in turn can be initiated to form type A cell types [Doetsch et al., 1999] (see Fig. 1.3 and Box I). In spite of the fact that SVZ-derived stem cells mainly differentiate into neuronal lineage, it has been shown that under certain circumstances these cells generate oligodendrocytes in the adult brain. Type B and transit-amplifying type C cells expressing Olig2 are able to give rise to non-myelinating NG2-positive and mature myelin-building oligodendrocytes after migration into the corpus callosum [Menn et al., 2006].

This multipotent capacity of aNSCs makes them an interesting endogenous alternative for new neurons and OLs after myelin loss such as occurs after damage or during the course of disease and they display therefore one of the main cells used during this thesis.

1.4 Myelin and disease

Evolution has resulted in bigger and more complex organisms with longer signalling pathways that had to be coordinated. A key for successful information processing techniques is the velocity of a propagated signal. The most important functions of myelin are to enhance nerve conduction and to protect the axon from damage. This increase in velocity is possible because the myelin sheath is not continuously wrapped around axons but interrupted at regular intervals. These non-myelinated interruptions are called

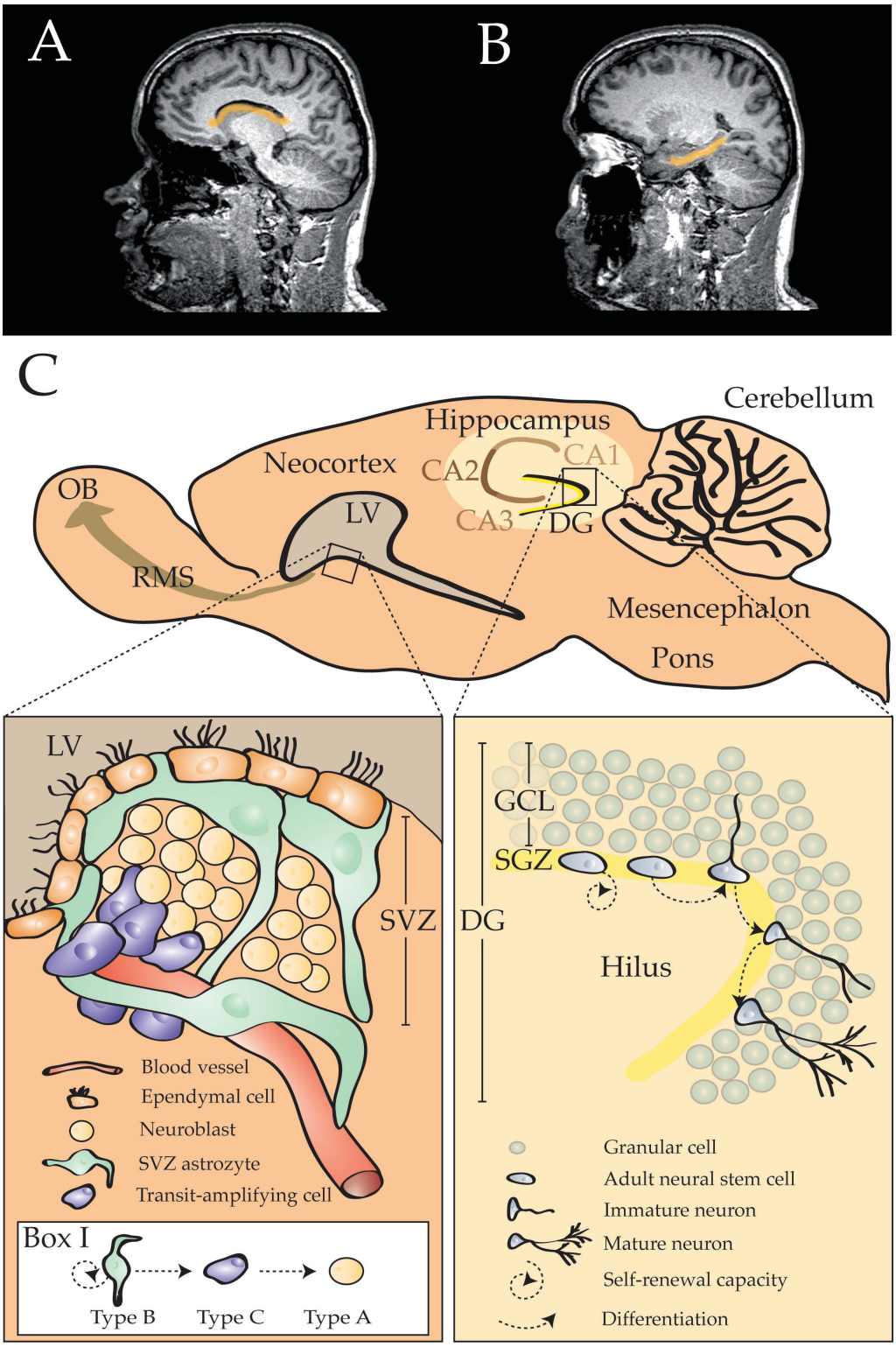

FIGURE 1.3: *Stem cell niches in the human and rodent brain*
The upper part of the illustration shows sagittal sections of the human brain. (**A**) The subventricular zone (SVZ) and (**B**) hippocampus are highlighted in yellow. (**C**) An illustrated sagittal section of the rodent brain shows to the left of the cerebellum the hippocampal formation with its subdivided units of the three cornu ammonis (CA1-

FIGURE 1.3: *Stem cell niches in the human and rodent brain* (continued)
CA3) areas and the dentate gyrus (DG) visible. The area in yellow next to the DG displays the subgranular zone (SGZ), where asymmetric and symmetric cell divisions of adult neural stem cells take place. A detailed magnification of the DG is shown in the bottom right box. To the left of the HC the lateral ventricle (LV) is illustrated residing below the neocortex. Higher magnification of the subventricular zone stem cell niche shows SVZ astrocytes, which — as adult neural stem cells — make contact with the ventricle and wrap an area containing neuroblasts and transit-amplifying cells. An ependymal cell layer lines the outer wall of that niche. The astrocytes additionally make contact with blood vessels for nutrient homeostasis. The progression of SVZ stem cells is shown in **Box I**. Type B SVZ astrocytes have a self-renewal capacity and are able to differentiate into type C transit-amplifying cells. Type C cells then may become type A neuroblasts and, for instance, begin migration along the rostral migratory stream (RMS) towards the olfactory bulb (OB).

nodes of Ranvier, where action potentials can occur. This isolation and the isolation gaps cause the action potential to "jump" from one node of Ranvier to the next, which is known as saltatory conduction. These "jumps" are responsible for the fast transmission of electric information and moreover reduce the energy demand for unnecessary action potentials.

Oligodendrocytes in the CNS and Schwann Cells in the PNS produce myelin, which consists of 40% water, 70–80% lipid dry mass and 15–30% protein [Boggs and Moscarello, 1978]. PNS and CNS myelin are quite similar but have minor differences in their structure as summarized by Baumann and Pham-Dinh. For instance, peripheral myelin protein 22 (PMP22) and P0 are not found in the CNS. P0 protein makes up a large proportion of PNS myelin, while CNS myelin is rich in MBP. Furthermore, MOG is unique in the CNS. Notably, myelin of OLs is highly enriched with glycosphingolipids such as GalC. However, the proportion of PLP in the CNS is much higher than compared with the proportion in the PNS. Oligodendrocytes start to produce myelin proteins when making contact with an axon. When contact between one of the ends of OLs and an axon is formed, the extension of the glial plasma membrane is wrapped around the axon [reviewed in Baumann and Pham-Dinh, 2001]. This wrapping is reminiscent of a whip cracking around the branch of a tree and tightening around it. Other theories suggest that myelin spirals around the axon from one side rather than spreading to both sites after attachment. Wrapped myelin surrounding an axon consists of multiple layers resembling the growth rings in the cross section of a tree.

1.4.1 Demyelination

High conduction velocity is important for the healthy adult nervous system. Diseases in which myelin is destroyed or damaged cause significant problems in everyday life and may eventually prove fatal. This demyelination process can be observed in multiple sclerosis (MS) [reviewed in Franklin, 2002]. MS is a chronic neuroinflammatory autoimmune disease that is associated with myelin loss and axonal degeneration. In this autoimmune disease, the adaptive immune system is misled and interprets the body's own epitopes as foreign (exogenous) and initiates a destructive immune response. Auto-reactive T-cells infiltrate the CNS and trigger an inflammatory reaction against myelin proteins and in certain cases possibly also against axons [reviewed in Lassmann et al., 2012]. Consequently, microglia and macrophages phagocytize myelin. In the course of MS, inflammatory lesions are found in the WM and GM. Additionally, neuronal loss occurs at early stages of the disease, resulting either indirectly from myelin loss or directly from immune cells attacking axonal structures. Because of the widespread distribution of lesions in the CNS, the symptoms of the disease vary from visual deficits or language disorders to severe cognitive deficits and paralysis.

1.4.2 Remyelination

In the course of demyelination and MS, spontaneous myelin recovery processes can be observed mostly during remission phases also known as remyelination [reviewed in Bunge et al., 1961; Prineas and Connell, 1979]. As long as oligodendroglial differentiation is a one-way event, meaning that mature OLs are not able to "de-differentiate" back into progenitor stages, these cells are not the key source of myelin repair during disease. Since OPCs are found in human MS lesions [Scolding et al., 1998], they may represent a "reservoir" for producing new myelin after damage to the CNS. Remyelination is thus mainly driven by CNS-resident progenitor cell activation and recruitment to lesions [reviewed in Franklin, 2002], also including aNSCs from stem cell niches [Menn et al., 2006; Picard-Riera et al., 2002]. Interestingly, human SVZ-derived stem cells are additionally capable of generating OPCs in active post-mortem human MS lesions [Nait-Oumesmar et al., 2007]. Nevertheless, the first activation and recruitment events assume that progenitor cells are in proliferative stages stimulated by endogenous growth factors [Redwine and Armstrong, 1998]. After migration and subsequent differentiation

of these NG2- and Olig2-positive progenitors into premyelinating OLs in lesions, they progress to mature OL stages and start wrapping myelin around demyelinated axons [Gensert and Goldman, 1997]. It is of note that not all resident OPCs become activated and repair demyelinated lesions but remain in a quiescent state rather than starting to proliferate [Wolswijk, 1998]. As a consequence, the number of OPCs does not increase and not enough myelinating OLs are produced, resulting in an inefficient remyelination process. Moreover, in every subsequent remyelination phase, the efficiency of successful restoration is diminished [Goldschmidt et al., 2009]. The recovery potential therefore decreases with disease progression. As an unfortunate result, such a remyelination phase cannot restore the entire myelin. In addition, newly formed myelin sheaths differ in thickness and length [Ludwin and Maitland, 1984]. Interestingly, these thin and short myelin structures are not found in healthy brains.
Nevertheless, the non-reversible loss of neuronal cells and the lack of an appropriate repair therapy are strong scientific motivation to investigate the nature of endogenous repair mechanisms in order to devise potential therapeutic approaches for neuronal as well as myelin (glial) repair.

1.4.3 Model to study demyelination and remyelination

A number of different animal models have been developed in order to study cellular and molecular processes related to pathology and regeneration in MS. An inflammatory reaction towards myelin in animals can be induced by myelin vaccination such as in the experimental autoimmune encephalomyelitis (EAE) model. EAE is initiated by injecting an organism's own myelin protein such as MBP, PLP or MOG, which results in auto-reactive immune cells directed against myelin structures. In the so-called MOG-EAE it was shown that immunization with MOG leads to the activation of naïve T-cells and then to a clonal production of myelin protein-reactive Type 1 helper T-cells (Th1-cells) [reviewed in Rangachari and Kuchroo, 2013]. After crossing the BBB, Th1-cells trigger pro-inflammatory cytokine secretion, which in turn initiates inflammation [Murphy et al., 2010]. Besides this actively triggered EAE, the passive application of already auto-reactive T-cells from donor mice into naïve animals underlines the autoimmune character of demyelinating diseases [Ben-Nun et al., 1981]. Since MOG-EAE is one of the best established and most frequently used MS models [Ben-Nun et al., 2006], we also use this model in our experiments. Nevertheless, as MS features a highly complex

pathogenesis and is also heterogeneous by nature, EAE only reflects a few pathogenic hallmarks.

1.5 Repair processes in diseased and injured CNS

At present, the question of how to deal with damage and injuries to the CNS is far from being solved. In view of the fact that cells die in many cases, either powerful neuro/glia protective treatments or cell replacement therapies must be designed. Exogenous cell therapy, in which transplantation of progenitor cells and neural stem cells leads to cell replacement, should be considered in this regard. At the same time, progenitor cells and neural stem cells, which reside in the tissue, are a potential source of endogenous repair. It may therefore be possible to promote endogenous repair mechanism activity by applying single (growth) factors or transplanted cells secreting a combination of factors.

1.5.1 Stimulation of endogenous repair mechanisms of the CNS

The observation that new neurons can be generated throughout human life in distinct areas of the adult human brain [Spalding et al., 2013] and, in the case of demyelinating diseases, that the CNS is capable of generating mature oligodendrocytes in lesions [reviewed in Bunge et al., 1961; Kotter et al., 2011] revives the hope that such endogenous mechanisms can be used to provide repair-mediating therapies leading to functional recovery following damage to and cell loss in the CNS. Factors involved in the recruitment of resident progenitor cells as well as adult stem cells are needed to improve endogenous repair mechanisms. One way of reaching this goal is by systematically applying specific factors. To do this, the respective packaging must cross the BBB and directly approach its target. For example, Fallon and colleagues showed a significant induction of proliferation and migration of SVZ-derived stem cells via transforming growth factor α (TGF-α) infusions [Fallon et al., 2000]. Furthermore, FGF-2 is also known to enhance proliferation and migration of adult neural stem cells, thereby activating neurogenesis [reviewed in Peterson, 2002]. However, it is difficult to devise such a strategy, as the potential application route crosses other tissues, which could be affected and cross-react with the factors. What is more, a direct infusion of factors into the brain is difficult to achieve.

Several studies have additionally described the therapeutic effects of transplanting cells to ameliorate endogenous cell repair. Here the therapeutic mechanism is rather based on factors secreted from transplanted cells than on the cell replacement capacity of transplanted cells. One of the most astonishing observations of recent years has been the influence of mesenchymal stem cells (MSCs) on aNSCs and oligodendroglial development. Fibroblast-like, "colony-forming" cells derived from guinea pigs cultivated on plastic surfaces [Friedenstein et al., 1970] was the first description of what was later classified as MSCs. Resident in the bone marrow, these cells have the ability to differentiate into various cell lineages as multipotent stem cells normally do. Hence, MSCs that have mesenchymal and mesodermal properties are distributed in mesenchymal tissues throughout the organism. These cells are responsible for the renewal of tissue in the aged body, after disease-induced damage or after accidents and can give rise to osteocytes, chondrocytes, adipocytes and blood cells.

MSCs were shown to improve survival of animals with neurodegenerative disorders [Jin et al., 2002] and appear to be safe for transplantation purposes [Mazzini et al., 2003]. Regarding the subject of this thesis it is worth mentioning that the co-culturing of aNSCs with MSCs results in direct NSC differentiation into oligodendrocytes [Rivera et al., 2006]. Elucidating that mechanism in greater depth, Rivera and colleagues demonstrated that MSC-conditioned medium (MSC-CM) contains MSC secreted factors which are responsible for this effect [Rivera et al., 2006]. Additionally, factors secreted by mesenchymal stem cells promote recovery in a MS model and enhance oligodendrogenesis as well as the generation of neurons by releasing hepatocyte growth factor (HGF) [Bai et al., 2012]. A detailed exploration of the MSC secreted factors remains an important future task. It has also been shown that MSCs exert immunosuppressive effects on T-cells and could therefore be used in anti-autoimmune therapies [Uccelli et al., 2006]. Interestingly, even bone marrow derived MSCs can improve the functionality of the spinal cord after contusion damage [Chopp et al., 2000].

Broadly speaking, MSCs represent a promising tool in inducing endogenous repair mechanisms in many disorders.

1.5.2 Exogenous cell replacement approaches

Cell transplantation into the CNS has been performed for quite some time now in order to elucidate cell survival, stimulation of axonal regeneration and functional interaction with

resident cells. This is a challenging approach, as it is not possible to transplant entire parts of the CNS, unlike other organs, due to its complex neuronal network. Scientists pursue exogenous cell transplantation approaches of well-defined CNS progenitors in order to determine to what extent a significant functional recovery, for example after spinal cord injury (SCI) [Groves et al., 1993], can be achieved.

For a long time, pluripotent embryonic stem cells (ESCs), which have the potential to differentiate into cells of all three germ layers, have been considered the "saviours" among the cell types in generating new tissue, and for this reason they are still a main focus when it comes to the development of new therapies for diseases. For instance, pathological conditions in which endogenous cell repair mechanisms are ineffective could be treated by ESC-based replacement strategies. Here, pluripotent stem cells could potentially contribute the required tissue-specific function — thus in contrast to resident cell types, which are no longer capable of doing so. Indeed, human embryonic stem cell-derived OPCs have also been transplanted into the myelin-lacking shiverer mouse model, which resulted in myelin formation [Nistor et al., 2005] and showed remyelination of axons after SCI [Keirstead et al., 2005]. While this requires a proper knowledge of the mechanisms involved in the differentiation processes of pluripotent stem cells, the use of ESCs is always attended by ethical concerns. ESCs are obtained from the inner cell mass of a blastocyst and therefore using them as a therapeutic tool raises serious ethical questions relating to their extraction, since the embryo dies in the process. In this context, an alternative approach has recently been shown to generate pluripotent stem cells out of fibroblasts by viral transduction of transcription factors [Takahashi and Yamanaka, 2006]. The reprogramming of cells into induced pluripotent stem cells (iPSCs) may be a way of avoiding the embryonic ethical question. It is sufficient in this case to obtain fibroblasts by skin biopsy, as opposed to killing an embryo. This procedure furthermore diminishes cell repulsion by an organism, as iPSCs are obtained from the host. It still remains to be fully ascertained whether iPSCs can be used as a complete or partial replacement for ESCs. Notably, a recent work described that "human iPSC-derived OPCs" are able to differentiate into myelin-forming OLs on transplantation into demyelinated lesions [Wang et al., 2013], therefore showing the reprogramming approach to have promising therapeutic potential. Nevertheless, one of the greatest disadvantages of pluripotent stem cells transferred or transplanted into new tissue is the increased risk of carcinomas [Nishimura et al., 2003].

Further, multipotent adult neural stem cells can give rise to a number of neural cell types when transplanted into different regions of the CNS. For instance, human neural stem cells have been described as strong candidates, as they display regular migration and differentiation following transplantation into the mouse brain [Flax et al., 1998]. In addition, it has been shown that hippocampal aNSCs, when expanded *ex vivo* and transplanted into the RMS, express tyrosine hydroxylase (TH), although TH expression is not present in the hippocampus [Suhonen et al., 1996]. This multipotent capacity is underlined by transplanting adult neural stem cells into neurogenesis-deficient tissue such as the striatum [Lindvall and Kokaia, 2006; Ziavra et al., 2012]. Here, aNSCs have the potential to form glial cells [Dziewczapolski et al., 2003], which can play an important role in replacing damaged cells after injury. Similar studies with human progenitors also show survival and differentiation into neurons and glial cells after transplantation [Svendsen et al., 1997].

However, one of the major limitations of cell replacement strategies is the glial differentiation of aNSC and the immune response after transplantation. In spite of aNSCs being able to stimulate and thereby modulate an immune cell response in order to therapeutically ameliorate cell replacement strategies after transplantation [Martino and Pluchino, 2006, 2007], local astrogenic cues are still an issue. Future cell replacement strategies therefore require pre-treatment of aNSC with anti-gliogenic factors to prevent astroglial differentiation after transplantation in order to enhance the development of neurons or oligodendrocytes respectively.

1.6 Cell fate decision and oligodendroglial differentiation in the adult CNS

Division of stem cells is either symmetric — increasing the number of stem cells — or asymmetric — in which one daughter becomes a specialized cell while the other retains stem cell characteristics. The decision of a neural stem cell to generate one of the described descendants — neurons, astrocytes or oligodendrocytes — is based on molecular signals determining their fate. Cell fate decision is orchestrated by many factors as well as by recently described mechanisms involving neuronal electrical inputs [Song et al., 2012]. For instance, neuronal determination has been observed under the influence of retinoic acid (RA), brain-derived neurotrophic factor (BDNF) or neurotrophin 3 (NT-3)

[Takahashi et al., 1999]. Conversely, this knowledge is also useful to understand oligodendroglia development, because pro-neuronal factors oppose glial development and may serve as targets for oligodendroglial regulation. Adult neural stem cells and OPCs are the main focus of cell-replacement-based therapies for demyelinating diseases such as MS on account of their much described crucial role in brain plasticity. This is why the underlying mechanisms involved in fate decision and differentiation of these cells are the subject of intensive investigation.

By way of clarification, the overexpression of Mash1 (also called as Ascl1) in collaboration with other bHLH TFs was found to induce neuronal differentiation in embryonic neural cells [Farah et al., 2000]. In the postnatal brain, however, Mash1 also regulates oligodendrogenesis of SVZ-derived aNSCs [Parras et al., 2004]. Moreover, Jessberger and colleagues demonstrated that a retroviral-based overexpression of Mash1 results in fate specification towards oligodendroglial lineage in the adult hippocampal stem cell niche of mouse and rat brains. While in the beginning (1d) Mash1-expressing aNSCs are not positive for the oligodendroglial marker NG2 and cannot be distinguished from controls by means of DCX, four days post retroviral application these cells mostly coexpress NG2 [Jessberger et al., 2008]. Interestingly, Mash1 is involved in oligodendroglial differentiation during remyelination, thereby positively influencing oligodendrogenesis during the course of disease [Nakatani et al., 2013]. Furthermore, as yet unknown environmental cues in the demyelination model seem to trigger oligodendroglial differentiation of resident SVZ aNSCs [Menn et al., 2006; Picard-Riera et al., 2002], growth factors may regulate this conversion [Aguirre et al., 2007; Ruffini et al., 2001]. The BMP antagonist chordin achieves this effect in corpus callosum after demyelination [Jablonska et al., 2010]. Therefore and as an important part of the scientific approaches of this thesis it is worth mentioning that BMPs play a crucial role in the complex tailored process of differentiation from a stem and progenitor status to a fully matured oligodendrocyte. For instance, the activation of Id2 and Id4 via BMPs blocks important transcription factors like Olig1 and Olig2 [Samanta and Kessler, 2004]. In detail, Id2 and Id4 bind to partners of the Olig transcription factors (E12 and E47), preventing Olig/E complexes. Olig1 and Olig2 are thus not able to translocate into the nucleus to activate pro-oligodendroglial gene transcription [Samanta and Kessler, 2004], and astrocyte formation is supported. Interestingly, the aforementioned oligodendroglial fate switch effect of MSC-CM on aNSCs is based on regulation of the ratio between Id and Olig proteins [Rivera et al., 2006; Steffenhagen et al., 2012]. Altogether, this shows

the complex orchestration of fate and decision-making in adult neural stem cells and progenitor cells.

1.7 Multifunctional potential of $p57^{Kip2}$

Preliminary work by our group has identified the cyclin-dependent kinase inhibitor 1C (CDKN1C) $p57^{Kip2}$ as a regulator of Schwann cell differentiation [Heinen et al., 2008a]. Initially, $p57^{Kip2}$ was identified as a negative regulator of cell proliferation. It was shown to be involved in the cell cycle and to inhibit different cyclin/CDK complexes in the transition of G1- to S-phase [Lee et al., 1995]. In studies with $p57^{Kip2}$ knockout mice, $p57^{Kip2}$ was shown to be indispensable for embryonic development. Knockout of $p57^{Kip2}$ leads to serious developmental disturbances and mice dying shortly after birth [Yan et al., 1997]. Besides its familiar role in the cell cycle, $p57^{Kip2}$ takes on other roles in cellular development. Its function in relation to cancer development is currently being investigated [Jiang et al., 1998; Jin et al., 2008; Larson et al., 2008; Shin et al., 2000; Yue and Jiang, 2005; Yue et al., 2003], but it has also been identified in developmental disturbances such as Beckwith-Wiedemann syndrome (BWS) in mice [Zhang et al., 1997]. This syndrome features asymmetric overgrowth and disproportionate size of body parts and organs. Additionally, the probability of forming embryonic tumours is highly increased in human children with BWS. $p57^{Kip2}$ has also been shown to directly regulate neuron differentiation, the specification of oligodendrocytes in zebrafish [Park et al., 2005], as well as to control the initiation of differentiation on cell cycle exit of mouse oligodendroglial cells in development [Dugas et al., 2007]. Joseph and colleagues have found that $p57^{Kip2}$ inhibits neuronal differentiation in embryonic neural stem cells in the rat brain [Joseph et al., 2009]. Moreover, downregulation of $p57^{Kip2}$ leads to functional compensation by the related protein $p27^{Kip1}$, a process involved in stem cell quiescence [Furutachi et al., 2013; Matsumoto et al., 2011; Zou et al., 2011]. Apart from the CDKI domain, $p57^{Kip2}$ contains a proliferating cell nuclear antigen (PCNA) binding domain that is required in the cell cycle [Watanabe et al., 1998]. In contrast to other Cip/Kip family members, $p57^{Kip2}$ also has a proline-rich LIM binding domain which enables $p57^{Kip2}$ to bind cytoplasmic kinase LIM kinase 1 (LIMK-1). LIMK-1 is known to regulate actin filament architecture and can be shuttled to the nucleus by $p57^{Kip2}$ [Yokoo et al., 2003]. In this context, our group also demonstrated a direct correlation

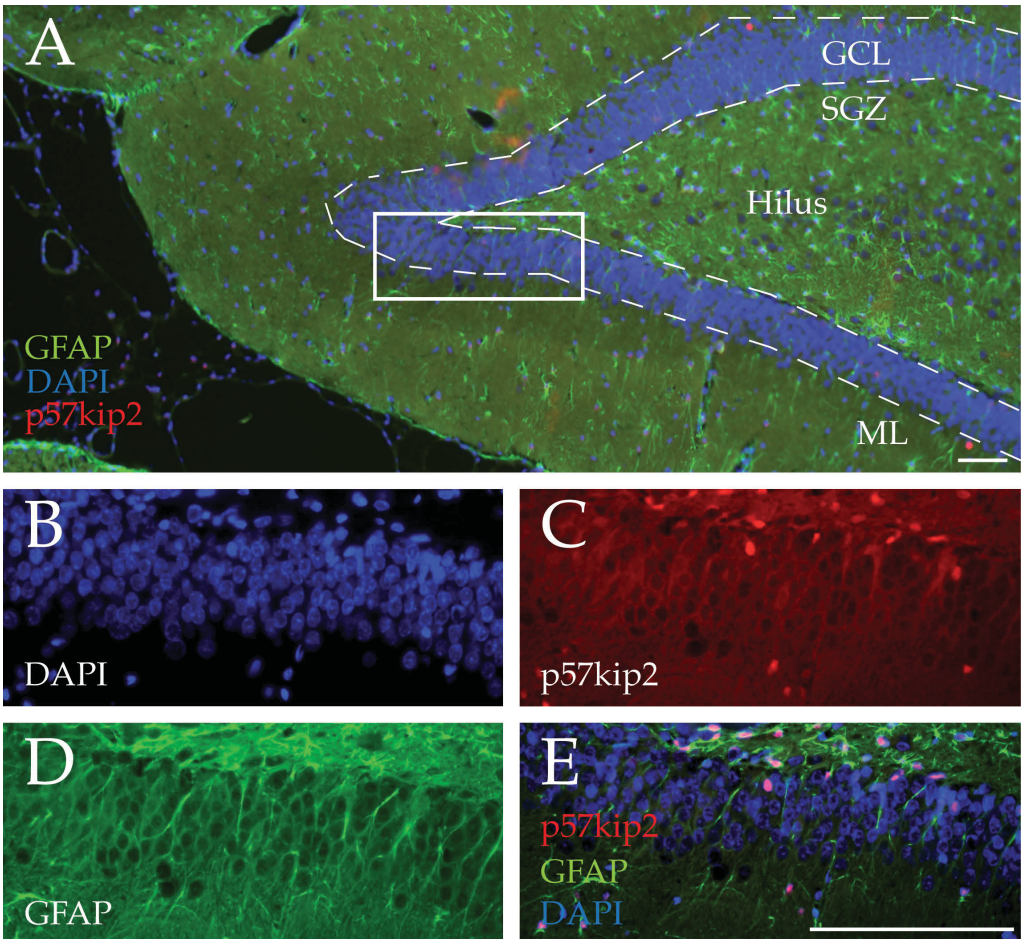

FIGURE 1.4: *Adult neural stem cells express p57^{Kip2}.*
Stainings for p57^{Kip2} and GFAP revealed p57^{Kip2}-positive cells in the sub granular zone (SGZ) of the hippocampal formation. The molecular layer (ML), granular cell layer (GCL) and SGZ enclose the hilus. Scale bars: 100 µm

between p57^{Kip2} and LIMK-1 in the peripheral nervous system [Heinen et al., 2008b]. At the beginning of this thesis, we were able to describe p57^{Kip2} protein levels in the SGZ (Fig. 1.4), which raises the question of whether p57^{Kip2} is also involved in differentiation of oligodendroglial progenitor cells and aNSCs in the CNS.

1.8 Aims of this thesis

Cell fate decision and a subsequent oligodendroglial differentiation of OPCs and aNSCs are highly complex orchestrating processes. Although oligodendroglial differentiation results in myelin-forming OLs in the adult CNS, these processes are impaired during disease and injury. Therefore, the aim of the research work presented here was to study

regulation patterns of glial fate decision processes, oligodendroglial differentiation as well as maturation in OPCs and aNSCs by modulating intrinsic differentiation programs, i.e. suppression of $p57^{Kip2}$ expression or under the influence of trophic factors secreted by bone marrow MSCs.

Based on these findings, the thesis sets out to address the following questions:

i) Is it possible to enhance oligodendroglial differentiation and maturation by intrinsic $p57^{Kip2}$ modulation or by extrinsic addition of mesenchymal stem cell secreted factors?

ii) Is it possible to increase the number of oligodendrocytes by modulating cell fate decision in progenitor cells and adult neural stem cells?

iii) Is it possible to prevent astroglial development?

Chapter 2

Publications — Results

2.1 $p57^{Kip2}$ is dynamically regulated in experimental autoimmune encephalomyelitis and interferes with oligodendroglial maturation

David Kremer, André Heinen, Janusz Joachim Jadasz, Peter Göttle, Kristin Zimmermann, Philipp Zickler, Sebastian Jander, Hans-Peter Hartung, and Patrick Küry

Abstract

The mechanisms preventing efficient remyelination in the adult mammalian central nervous system after demyelinating inflammatory diseases, such as multiple sclerosis, are largely unknown. Partial remyelination occurs in early disease stages, but repair capacity diminishes over time and with disease progression. We describe a potent candidate for the negative regulation of oligodendroglial differentiation that may underlie failure to remyelinate. The $p57^{Kip2}$ gene is dynamically regulated in the spinal cord during MOG-induced experimental autoimmune encephalomyelitis. Transient down-regulation indicated that it is a negative regulator of post-mitotic oligodendroglial differentiation. We then applied short hairpin RNA-mediated gene suppression to cultured oligodendroglial precursor cells and demonstrated that down-regulation of $p57^{Kip2}$ accelerates morphological maturation and promotes myelin expression. We also provide evidence

that $p57^{Kip2}$ interacts with LIMK-1, implying that $p57^{Kip2}$ affects cytoskeletal dynamics during oligodendroglial maturation. These data suggest that sustained down-regulation of $p57^{Kip2}$ is important for oligodendroglial maturation and open perspectives for future therapeutic approaches to overcome the endogenous remyelination blockade in multiple sclerosis.

Approximated total share of contribution: 10%

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Contribution on experimental design, realization and publication
Oligodendrocyte culture, transfection, stainings and data analysis were performed by Janusz Joachim Jadasz. The manuscript including all figures was subsequently reviewed, amended and approved as co-author.

Data used for other degree than this thesis
The data presented in this publication has previously been submitted for the medical doctoral degree of Dr. David Kremer at the Heinrich-Heine-Universität Düsseldorf.
`http://docserv.uni-duesseldorf.de/servlets/DocumentServlet?id=13597`

Link to the publication
`http://www.pnas.org/content/106/22/9087.long`

p57kip2 is dynamically regulated in experimental autoimmune encephalomyelitis and interferes with oligodendroglial maturation

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The mechanisms preventing efficient remyelination in the adult mammalian central nervous system after demyelinating inflammatory diseases, such as multiple sclerosis, are largely unknown. Partial remyelination occurs in early disease stages, but repair capacity diminishes over time and with disease progression. We describe a potent candidate for the negative regulation of oligodendroglial differentiation that may underlie failure to remyelinate. The p57kip2 gene is dynamically regulated in the spinal cord during MOG-induced experimental autoimmune encephalomyelitis. Transient down-regulation indicated that it is a negative regulator of post-mitotic oligodendroglial differentiation. We then applied short hairpin RNA-mediated gene suppression to cultured oligodendroglial precursor cells and demonstrated that down-regulation of p57kip2 accelerates morphological maturation and promotes myelin expression. We also provide evidence that p57kip2 interacts with LIMK-1, implying that p57kip2 affects cytoskeletal dynamics during oligodendroglial maturation. These data suggest that sustained down-regulation of p57kip2 is important for oligodendroglial maturation and open perspectives for future therapeutic approaches to overcome the endogenous remyelination blockade in multiple sclerosis.

differentiation | intrinsic inhibitor | multiple sclerosis | oligodendrocyte | remyelination

Multiple Sclerosis (MS) is the most common inflammatory demyelinating disease of the human central nervous system (CNS), featuring gradual degeneration and loss of previously established functional myelin sheaths and, eventually, of oligodendrocytes. As a consequence, saltatory nerve conduction is impaired, and axons are damaged (1, 2). The disease course can be highly variable, and most patients first experience recurrent and reversible neurological symptoms (relapsing-remitting MS) before a transition to secondary progressive MS occurs (3).

Oligodendrocytes, as the myelin-producing cells of the CNS, are key target cells in MS. Immune-mediated attack on the myelin sheaths leads to functionally impaired glial cells and may induce oligodendroglial death (4). Despite a generally limited regeneration capacity of the adult CNS, remyelination does occur, particularly in early disease stages, and can lead to functional improvement most likely resulting from activation of resident oligodendrocyte precursor cells (OPCs) that can differentiate into functional myelinating cells (5). Nevertheless, compared with peripheral nervous system (PNS) lesions in which remyelination of nerve fibers is widely observed, remyelination of CNS lesions is insufficient and rarely leads to a complete clinical remission. This insufficiency may result from the limited size of the OPC pool in the CNS as well as incomplete OPC activation and/or differentiation.

Several lines of evidence suggest that deficient remyelination may be a result of blocked cellular differentiation (6, 7); the transcriptional regulators, Hes1, Hes5, Id2, and Id4, the Notch signaling pathway, and the transmembrane protein LINGO-1 all negatively influence oligodendroglial differentiation (8–14). However, the expression of inhibitory factors might be stage specific, with the differentiation process regulated differently for early progenitors versus adult resident precursor cells. This circumstance suggests that remyelination in adults is a process different from myelination during development, reflecting differences between perinatal and adult OPCs (15). In addition, normal developmental processes do not occur in the background of an inflammatory disease, which further highlights the need to explore remyelination in adult MS-related model systems.

In this study, we demonstrate that the p57kip2 gene is regulated during the course of MOG-induced experimental autoimmune encephalomyelitis (EAE), a model that shares many pathological features with MS, including partial remyelination (16, 17). Its observed transient down-regulation before disease remission suggests it to be an intrinsic inhibitor of oligodendroglial (re)differentiation. As a member of the cip/kip family, p57kip2 was originally described as a cyclin-dependent kinase inhibitor (CDKI), but a number of other cellular processes were recently shown to depend on cip/kip proteins (18). For instance, long-term suppression of p57kip2 in Schwann cells, the myelinating glial cells of the PNS, efficiently induces their differentiation in culture, even in the absence of axons (19). We therefore investigated the impact of down-regulated p57kip2 levels on oligodendroglial maturation and report here that this results in acceleration of cellular differentiation in vitro. These findings may influence our understanding of naturally occurring remyelination, and down-regulation of the p57kip2 gene could provide the basis for remyelination therapies.

Results

Dynamic Regulation of p57kip2 Expression in the Diseased Spinal Cord. We performed quantitative RT-PCR analysis of spinal cord RNA to determine changes in p57kip2 expression during the course of EAE induced by immunization of DA rats with MOG protein. Most prominent was a strong down-regulation of p57kip2 expression during the first bout (at 11 days) and a moderate down-regulation during the second bout (at 22 days), indicating that lowered p57kip2 expression levels correlate with the onset of remission (Fig. 1 *A* and *B*). Beyond 28 days, no further regulation of p57kip2 expression was observed. Immunostaining revealed many p57kip2 expressing cells throughout the healthy spinal cord (Fig. 1*C*). EAE-related inflammation resulted in decreased transcript levels reflected by a lower number of p57kip2-positive cells (shown for 11 days after MOG immunization in Fig. 1*D*). Interestingly, we observed that the

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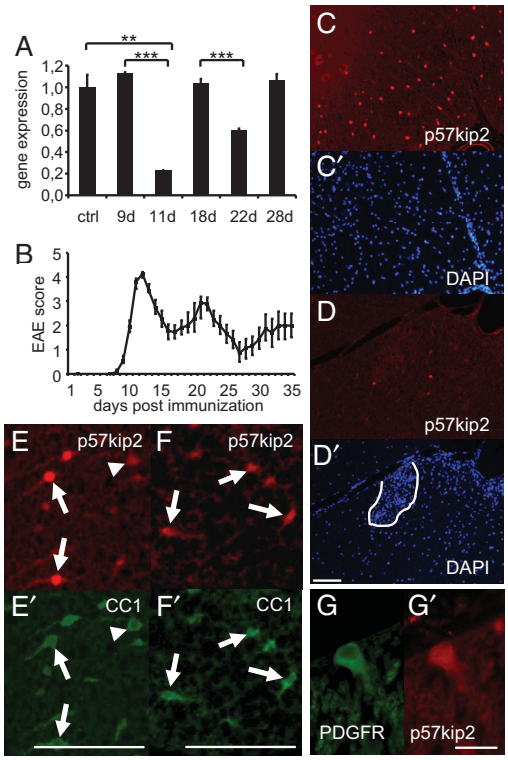

Fig. 1. Regulation of p57kip2 expression in MOG-EAE spinal cords. (*A*) Quantitative RT-PCR analysis of p57kip2 expression in the diseased spinal cord. Down-regulation is observed at the first and, to a lesser degree, at the second bout, as revealed by the peak EAE scores near days 11 and 22 shown in *B* [One of 3 independent measurements is shown; GAPDH expression was used as reference and data are mean values ± SEM (*t* test: **, $P < 0.01$, ***, $P < 0.001$)]. (*C–D′*) Anti-p57kip2 immunostainings and DAPI counter stains of healthy spinal cord (*C* and *C′*) and 11-day MOG-EAE spinal cord (*D* and *D′*) sections; dotted line in *D′* marks infiltrating immune cells. Double immunostainings for p57kip2 and CC1 (*E* and *E′*) and for p57kip2 and PDGFR-α (*G* and *G′*) indicating that within the healthy spinal cord white matter, p57kip2-expressing cells are oligodendrocytes and oligodendroglial precursor cells. Note that in the healthy tissue the majority of cells feature strong nuclear p57kip2 expression (arrows), whereas few cells show low cytoplasmic/perinuclear expression levels (arrowhead). At the end of the second bout (24 days), CC1-positive cells were found to express p57kip2 again (*F* and *F′*). [Scale bars: 100 μm (*D′*, *E′*, and *F′*); 20 μm (*G′*).]

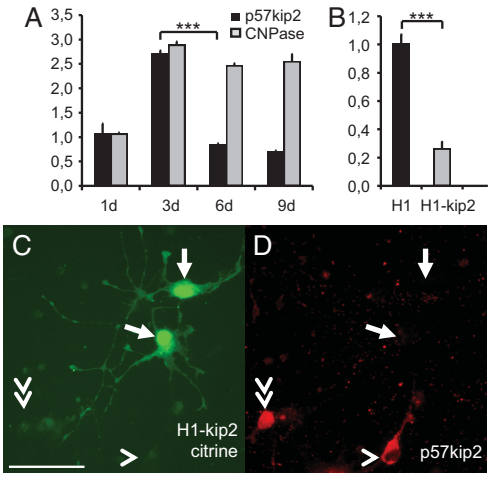

Fig. 2. p57kip2 regulation in cultured oligodendroglial cells. (*A*) Quantitative RT-PCR analysis revealed an initial gene induction followed by down-regulation of p57kip2 expression during the oligodendrocyte differentiation process which is accompanied by induction of CNPase expression (d, days in culture). (*B*) This analysis also confirmed p57kip2 down-regulation in suppressed and sorted oligodendroglial cells [One representative experiment of 5 is shown; GAPDH expression was used as reference and data are mean values ± SEM (*t* test: ***, $P < 0.001$); H1, control transfected cells; H1-kip2, p57kip2-suppressed cells]. (*C* and *D*) Anti-p57kip2 immunostaining (*D*) demonstrated that 6 days following transfection, p57kip2-suppressed-OPCs (*C*; marked by expression of citrine) were still devoid of p57kip2 expression. The arrows point to transfected cells, the arrowhead marks a cell with p57kip2 signals outside the nucleus, and the double arrowhead points to a cell with strong nuclear p57kip2 expression. [Scale bar, 50 μm (*C*).]

reduction of the p57kip2 signal was overall and not dependent on direct contact with infiltrating immune cells (dashed line in Fig. 1*D′*). In the white matter of healthy adult spinal cord (Fig. 1 *E* and *E′*) and at the end of the second bout (24 days after MOG immunization; Fig. 1 *F* and *F′*), p57kip2-positive cells were mostly CC1-positive oligodendrocytes. A lower number of p57kip2-positive cells expressed the oligodendrocyte precursor marker platelet-derived growth factor receptor-α (PDGFR-α; Fig. 1 *G* and *G′*). We detected no strong p57kip2 signals in infiltrating immune cells (Fig. 1*D′*) and no overlap with GFAP signals, indicating that astrocytes did not contribute to the p57kip2 signals. Of note, in the healthy spinal cord the majority (>90%) of p57kip2-expressing cells displayed strong nuclear as well as perinuclear p57kip2 signals (Fig. 1*E*, arrows), whereas a lower number of cells showed only cytoplasmic/perinuclear signals of weaker intensity (Fig. 1*E*, arrowhead). This latter subpopulation was substantially increased in the diseased spinal cord (Fig. 1*F* and *F′*), indicating that p57kip2 down-regulation might be accompanied by protein relocation. The observation that p57kip2 down-regulation fades over time can therefore be interpreted as this gene acting as oligodendroglial inhibitor in (advanced) pathophysiological situations.

Regulation of p57kip2 Expression in Cultured Oligodendroglial Cells. As a next step, we investigated expression and regulation of p57kip2 in cultured primary oligodendroglial cells derived from newborn rat cortices. Oligodendrocyte precursor cells (OPCs) were induced to differentiate in culture by means of growth factor withdrawal and then analyzed for gene and protein expression. Determination of gene expression levels by means of real-time quantitative RT-PCR demonstrated an initial increase of p57kip2 expression concomitant with cell cycle exit and differentiation onset followed by decreasing transcript levels during the course of cellular maturation (Fig. 2*A*). This process was accompanied by a moderate induction of CNPase- (Fig. 2*A*) as well as a strong induction of MBP expression. A similar biphasic expression profile has already been described for cultured OPCs derived from P7 optical nerves (20).

Following the observation that p57kip2 is down-regulated in both the remyelinating spinal cord as well as in differentiating OPCs, we determined to what degree p57kip2 gene suppression affects the cellular differentiation process. To this end we applied RNA interference to decrease this gene's activity in cultured oligodendroglial cells. However, silencing RNA (siRNA) dependent approaches led only to transient suppression of p57kip2 expression. As an alternative, we used a small hairpin RNA (shRNA) cassette (19), which allows long-term p57kip2 suppression and was previously shown to be specific for p57kip2 and free from off-target effects, such as an IFN response

induction (19). By using this approach, oligodendroglial p57kip2 expression could be reduced for up to 9 days as shown by quantitative RT-PCR of transfected and sorted OPCs 2 days following transfection (Fig. 2*B*) as well as by anti-p57kip2 immunostaining 6 days following transfection (Fig. 2 *C* and *D*). Note that among cultured OPCs, cells with strong nuclear p57kip2 expression (double arrowhead) as well as cells with perinuclear and cytosolic expression only (arrowhead) were detected.

Reduced p57kip2 Levels Lead to Accelerated Oligodendroglial Differentiation. A number of different pathways are known to promote oligodendroglial differentiation featuring different patterns of gene expression (21, 22). Growth factor withdrawal results in immediate cell cycle exit and differentiation onset, whereas environmental cues such as retinoic acid or thyroid hormone appear to depend on an intracellular molecular clock (23, 24). To determine to what degree decreasing levels of p57kip2 affect differentiation kinetics, we used mitogen withdrawal to study maturation of post-mitotic cells and determined the distribution of cellular morphologies of control-transfected and p57kip2-suppressed oligodendroglial cells (Fig. 3). For visualization, OPCs were co-transfected with a citrine expression vector as described previously (19). Differentiation of cultured OPCs is not synchronized and thus is seen as a heterogeneous population of cells with various degrees of maturation, featuring increasing numbers of processes and secondary branches. In our analysis, we distinguished 6 different morphologies (see bar at the bottom of Fig. 3*E*) from a "very low" number of processes in progenitor cells to multiple process-bearing cells ("low," "medium," and "high") to mature cells with a "very high" degree of arborization or a flattened appearance ("sheaths"). Twenty-four hours after transfection, PDGF-AA and bFGF were withdrawn from the culture, and OPCs were exposed to low serum containing medium stimulating differentiation. After 24 h in this differentiation-promoting medium, p57kip2-suppressed cells appeared to be slightly advanced in their morphological maturation. However, at later time points (3, 6, and 9 days post-differentiation onset), a strong acceleration of morphological maturation was observed in p57kip2 suppressed cells (gray bars) compared with control transfected cells (black bars; Fig. 3 *C*–*E*). This maturation-promoting effect was also observed when additional myelin-enhancing stimuli were provided such as thyroid hormones and/or ciliary neurotrophic factor (CNTF) (12, 20, 25) or in presence of growth factors, indicating that this differentiation-promoting effect is a specific consequence of lowered p57kip2 levels.

Oligodendroglial maturation is also reflected by the induction and expression of specific marker proteins. We determined whether the pattern of marker expression was altered on p57kip2 suppression (Fig. 4). Following transfection and subsequent induction of cellular differentiation, control and p57kip2-suppressed cells were fixed at various time points and subjected to immunofluorescent staining with antibodies directed against the early marker O4 as well the myelin proteins CNPase (2′,3′-cyclic nucleotide 3′-phospho-diesterase), MBP (myelin basic protein), and MOG (myelin oligodendrocyte glycoprotein). We demonstrated that down-regulation of p57kip2 leads to a significant induction of oligodendrocyte markers at all time points investigated and shown for CNPase, O4, and MBP 2 days and MOG 4 days following initiation of cell differentiation (Fig. 4). Similar to the morphological analysis presented above, this marker induction was observed under a variety of culture conditions stimulating OPC differentiation.

p57kip2 Overexpression Can Induce LIMK-1 Nuclear Translocation. It has previously been shown that the p57kip2 protein can directly interact with LIM domain containing binding partners such as LIMK-1 (26), and we gathered preliminary evidence that in Schwann cells such specific protein/protein contacts can occur (27). Given that cytosolic LIMK-1, through phosphorylation of

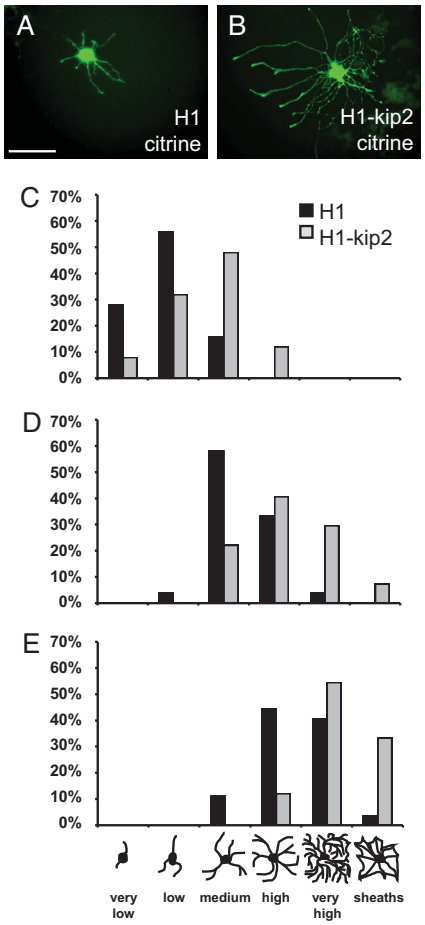

Fig. 3. p57kip2 suppression accelerates morphological maturation of cultured oligodendroglial cells. (*A* and *B*) Representative citrine-positive control-transfected (*A*) and p57kip2-suppressed OPCs (*B*) 3 days following induction of differentiation, is shown. The control transfected cell (H1) features a medium, whereas the p57kip2-suppressed cell (H1-kip2) features a high degree of morphological maturation. (*C*–*E*) Determination of OPC morphology distribution of control-transfected (H1; black bars) versus p57kip2-suppressed (H1-kip2, gray bars) cells at 3 (*C*), 6 (*D*), and 9 days (*E*) post-differentiation onset. At all time points, p57kip2-suppressed cells were morphologically advanced as compared with control-transfected cells. Six different morphologies were distinguished; 1 representative experiment of 7 is shown. [Scale bar, 50 μm (*A*).]

cofilin, can affect actin filament stability, hence, cell shape and motility, such an interaction might be part of p57kip2's mode of action in inhibiting oligodendroglial differentiation. We therefore investigated the subcellular localization of LIMK-1 in cultured OPCs. In addition, we evaluated the impact of p57kip2 overexpression on an existing subcellular LIMK-1 distribution (Fig. 5). OPCs were either co-transfected with a p57kip2-overexpressing or an empty control vector together with the citrine expression vector (19). Differentiation promoting medium was added and, after 48 h cells, were scored for LIMK-1 subcellular localization by means of immunofluorescence staining. In nontransfected and control-transfected cells, 2 subpopulations of LIMK-1 expressing OPCs were observed. In the majority of cells, signals could be detected in cellular processes and soma (including cell nucleus; Fig. 5 *C* and *C*′), whereas a

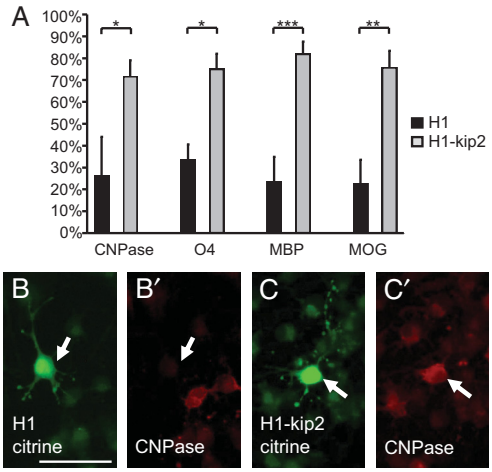

Fig. 4. p57kip2 suppression stimulates oligodendroglial marker expression. (*A*) Two days following differentiation induction, significantly more p57kip2-suppressed cells (H1-kip2; gray bars) expressed the early oligodendroglial marker O4 and the myelin proteins MBP and CNPase compared with control-transfected (H1; black bars). Similarly, 4 days post-differentiation onset, the number of MOG expressing cells was increased [One representative experiment of 9 shown; data are mean values ± SEM (*t* test: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$)]. (*B* and *B′*) Representative examples of CNPase-negative control-transfected (H1) and (*C* and *C′*) CNPase-positive p57kip2-suppressed (H1-kip2) cells are shown. Arrows point to transfected and citrine positive cells. [Scale bar, 50 μm (*B*).]

minority of cells displayed strong nuclear signals and reduced LIMK-1 in processes (Fig. 5 *B* and *B′*). Following p57kip2 overexpression, a significant increase in the number of cells with nuclear signals could be observed (Fig. 5*A*), indicating that in oligodendroglial cells p57kip2 can translocate LIMK-1.

Discussion

We demonstrate that expression of the p57kip2 gene is transiently down-regulated under inflammatory, demyelinating, pathophysiological conditions before the onset of disease remission. Mimicking down-regulation by means of long-term shRNA dependent suppression demonstrated that differentiation of oligodendroglial cells is accelerated in response to lowered p57kip2 levels. Our previous studies revealed a similar function in the related Schwann cell lineage (19), and together these data strongly suggest that p57kip2 encodes an intrinsic inhibitor of myelinating glial cell differentiation.

Whereas cultured Schwann cells are not able to differentiate spontaneously, at least when p57kip2 levels are unchanged, they readily do so in the lesioned or diseased PNS, which contributes to successful nerve repair (28). Cultured OPCs can differentiate but in vivo their capacities to adapt and to de- and redifferentiate are highly limited, resulting in conduction deficits and subsequent axonal degeneration (1, 2). Apart from inhibitory influences from the surrounding tissue, intrinsic blockades such as that provided by p57kip2 may also account for these differences between glial cell types (6, 7) and the ability to control inhibitor expression levels may dictate whether remyelination and repair can occur. Considering that substantial regulation by p57kip2 is only seen in early disease stages, it could be responsible for the limited regeneration capacity of the inflamed CNS seen later in disease. These data all suggest that disease-stage specific regulation of p57kip2 could constitute a promising approach for future remyelination therapies.

In a recent study on cultured P7-P8 optical nerve oligodendrocytes, it was shown that the induction of p57kip2 is a component of the intracellular timer mechanism that controls the differentiation onset in the presence of mitogens and on specific differentiation cues (20). We here demonstrate that the secondary decrease in p57kip2 expression is functionally coupled to the maturation process and does not only constitute the downside of the peak expression. It therefore appears that p57kip2 controls oligodendrocyte differentiation at various levels and via several pathways. The extent of up- and down-regulation is probably dependent on the OPC source and differentiation state [e.g., whether or not additional selection markers such as O4 (20) were used]. Such differences might even be more widespread among the different oligodendroglial lineages (29) and could include contributions from p21cip1 and p27kip1 (30, 31). However, precursor cell cycle control in the adult CNS appears not to depend on p57kip2 induction, because up-regulation was clearly not detected during the course of MOG-induced EAE in our studies. It is therefore possible that such a (p57kip2-dependent) timer function does not act on adult OPC differentiation and that adult remyelination is primarily regulated by epigenetic mechanisms (32).

In contrast to our data, Dugas and colleagues (20) reported a negative effect on OPC differentiation of siRNA-dependent suppression in presence of growth factors, an effect which was substantially reduced on withdrawal of growth factors. Under these conditions, combined suppression of p57kip2 and p27kip1 appeared to have a much larger effect, supporting the idea that several CDKIs act together. Our findings, on the other hand, show that p57kip2 suppression promotes maturation (Figs. 3 and 4). Although it cannot be excluded that OPCs from different sources respond differently to RNA interference, this difference could be simply technical because we were neither able to stably suppress p57kip2 expression by using siRNAs in primary Schwann cells (19) nor in OPCs, and instead used shRNA-encoding constructs. However, for both procedures, we used identical interference sequences recognizing all known cDNA sequences of the rat p57kip2 gene. This DNA approach was previously shown to be specific for p57kip2 and free from off-target effects (19) and we verified that suppression was achieved and also maintained (Fig. 2). It thus appears that a sustained down-regulation is imperative for an effect on OPC differentiation and that the vector based shRNA approach is highly efficient.

Because we found that long-term p57kip2 suppression accelerates differentiation parameters under a number of different

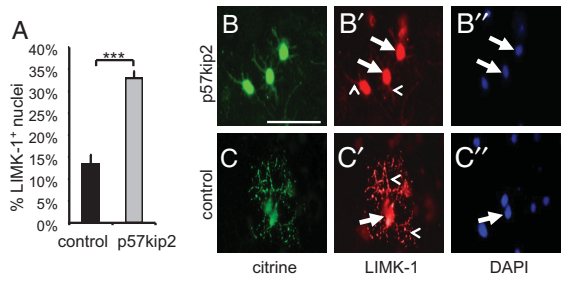

Fig. 5. p57kip2 overexpression leads to nuclear accumulation of LIMK-1. (*A*) Quantitative determination of the degree of nuclear LIMK-1-expressing oligodendrocytes in control-transfected (black bar) versus p57kip2-overexpressing OPCs (gray bar) [One representative experiment of 2 shown; data are mean values ± SEM (*t* test: ***, $P < 0.001$)]. (*B* and *C*) Anti-LIMK-1 immunostainings of cultured oligodendroglial cells 2 days following differentiation onset are shown. (*C*) Most of the control-transfected cells showed a widespread LIMK-1 expression. (*B*) On p57kip2 overexpression, more cells showed strong nuclear and reduced or absent LIMK-1 signals in processes. Arrows mark nuclear and arrowheads mark localization in processes. [Scale bar, 50 μm (*B*).]

culture conditions, including that used for P7-P8 optical nerve cells (20), it is unlikely that functional differences were because of different culture media compositions.

A general assumption is that mature oligodendrocytes are not able to de- and redifferentiate and that therefore remyelination must be a consequence of resident precursor cell activation. It was therefore surprising to see that almost all oligodendroglial cells within the spinal cord clearly down-regulated p57kip2, independent on whether they were mature or precursor cells or whether they were close to immune infiltrates. In light of this strong overall regulation in early phases of MOG-induced EAE, one could speculate that in such situations the majority of cells at least attempt to attain a cellular state during which redifferentiation and repair is facilitated. Whether the observed overall down-regulation of p57kip2 in early phases allows both cell types (resident precursor cells as well as CC1-positive oligodendrocytes) to adapt, at least partially, with precursor cells being more efficient or successful in executing differentiation, is currently unknown and awaits functional experiments in vivo.

Down-regulation of p57kip2 in vivo appears to be a consequence of inflammation. Nevertheless, our data suggest that either the signaling molecules controlling p57kip2 expression differ at different stages of disease or that the oligodendroglial capacity to respond diminishes over time. Either mechanism could be responsible for the observation that less regulation can be seen in later attacks and during progression to more chronic stages. It is also not known what signals account for this overall p57kip2 regulation during inflammation. The identification of such presumably diffusing regulatory factors could promote the development of p57kip2-based remyelination therapies, and thus provide an alternative approach to the currently available in vivo gene suppression approaches (33).

Our overexpression studies indicate that p57kip2 interacts with LIMK-1. It is therefore conceivable that subtle changes of p57kip2 levels, such as on down-regulation in vivo or in cultured cells, could regulate the subcellular distribution of LIMK-1 affecting the equilibrium of enzymes and regulators that control cytoskeletal dynamics (34). Down-regulation of p57kip2 could thus moderately increase cytosolic levels of LIMK-1, leading to increased cofilin phosphorylation and inactivation and thus promote actin filament growth and stabilization, resulting in the observed oligodendroglial morphological changes. It is tempting to speculate that such cell specific regulations of ubiquitously expressed enzymes might be involved in cell differentiation and regeneration as they occur in demyelinating diseases. Whether or not p57kip2 is the only means to control LIMK-1 activity remains to be addressed by future experiments. In addition, future experimental approaches will also be needed to understand how down-regulation of a single gene can affect multiple cellular parameters. These studies will reveal the extent of induced alterations in gene expression, whether or not they are secondary to morphological events and whether interactions with related LIM proteins account for these additional aspects of glial differentiation.

In summary, we have shown that a sustained reduction of p57kip2 expression levels in oligodendroglial cells facilitates and promotes their differentiation. This observation indicates that p57kip2 is a regulator of (re)myelination at the interface between morphogenesis and gene expression. This outcome is of particular interest regarding our still-limited understanding of (re)myelination mechanisms and will be important in defining novel strategies to promote endogenous remyelination and CNS repair. In this regard, it will be imperative to determine to what degree p57kip2 expression directly affects (re)myelination in vivo. Because p57kip2 knockout mice suffer from a number of tissue defects and die around birth (35), such functional studies can only been carried out once suitable mouse mutants have been generated.

Materials and Methods

MOG-Induced Experimental Autoimmune Encephalomyelitis (MOG-EAE). All rat experiments were performed in accordance with institutional guidelines. MOG-EAE was induced in 10- to 14-week-old female DA ($RT1^{av1}$) rats (Harlan) by active immunization with the recombinant MOG protein corresponding to the N-terminal sequence of rat MOG (amino acids 1–125) in complete Freund's adjuvans, as described previously (36). The clinical status of the animals was scored as follows: 0 = no clinical signs; 1 = loss of tail tone; 2 = complete tail paresis; 3 = hind limb weakness; 4 = complete hind limb paraplegia; 5 = tetraparesis; 6 = moribund state; and 7 = death. Animals were followed for a maximum of 35 days and developed a multiphasic disease course with 2 episodes reaching their peak at days 11–12 and 21–22, respectively (see Fig. 1*B*). For isolation of total RNA, animals were killed at days 9, 11, 18, 22, and 28 after immunization ($n = 3–4$ per time point), corresponding to onset and peak of the first (days 9, 11) and second episode (days 18, 22), and the time point of remission from the second episode (day 28), respectively. As controls, naïve nonimmunized DA rats ($n = 3$) were used.

Oligodendroglial Cell Culture. Purification and culturing of OPCs was performed according to (37). Briefly, dissociated P1 rat cortices were cultured on polyD-lysine (PDL)-coated cell culture flasks in DMEM substituted with 10% FCS and 4 mM L-glutamine. After 10 days, flasks were shaken at 250 rev/min for 2 h to deplete from microglial contamination. Then flasks were shaken for another 20 h in which OPCs were dislodged from the underlying astrocyte-layer and replated on PDL-coated culture dishes or glass cover slips in high glucose DMEM-Sato-based medium containing bovine 5 μg/mL insulin, 50 μg/mL human transferrin, 100 μg/mL BSA, 6.2 μg/mL progesterone, 16 μg/mL putrescine, 5 ng/mL sodium selenite, and 4 mM L-glutamine (all Sigma). Anti-A2B5 staining revealed that at this point the cultures consisted of 98% oligodendroglial cells. OPCs were either kept in proliferation medium (Sato medium with 10 ng/mL bFGF and 10 ng/mL PDGF-AA; R&D Systems and Peprotech), whereas differentiation was initiated by Sato medium which was depleted from growth factors and supplemented with either 0,5% FCS, 10 ng/mL CNTF (Chemicon) or 400 ng/mL T3/T4 thyroid hormones (Sigma). Generation and transfection of pSUPER based suppression vectors (OligoEngine) or pIRES2EGFP based expression vectors (BD Biosciences) were described previously (19). Isolation of citrine positive OPCs was done by fluorescence activated cell sorting (FACS Aria, BD Biosciences).

Immunostaining. Immunostaining on paraffin sections from paraformaldehyde-perfused rat spinal cords or paraformaldehyde-fixed cultured cells was performed as described previously (19). Primary antibodies were diluted as follows: rabbit anti-p57kip2 antibody (1/200; Sigma-Aldrich), mouse anti-APC/CC1 (1/1,000; Calbiochem), mouse anti-O4-, mouse anti-PDGFR-α-, mouse anti-A2B5 antibodies (1/100, 1/300, and 1/200, respectively; all Chemicon), mouse anti-MOG antibody (1/1,000; B. Hemmer), mouse anti-MBP- and mouse anti-CNPase antibodies (1/1,000 and 1/500, respectively; both Sternberger Monoclonals) and rabbit anti-LIMK-1 (1/500; BD Biosciences). Alexa Fluor 488-, Alexa Fluor 594-, or horseradish peroxidase-conjugated antibodies (1/500; all Molecular Probes) were used for signal visualization. Nuclei were stained with DAPI (Roche).

RNA Preparation, cDNA Synthesis, and Quantitative RT-PCR. For purification of total RNA from spinal cord and cultured cells, we used the TRIzol reagent (Invitrogen) and the RNeasy procedure (Qiagen), respectively, following the protocols of the suppliers. Isolated RNA was reverse transcribed by using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative determination of gene expression levels was performed on an ABI 7000 sequence detection system by using Power SybrGreen universal master mix (Applied Biosystems). Primer sequences were determined by means of PrimerExpress 2.0 software (Applied Biosystems) and subsequently tested for the generation of specific amplicons (for sequences see ref. 19). GAPDH and ODC were used as reference genes, and relative gene expression levels were determined according to the manufacturer's $\Delta\Delta$Ct method (Applied Biosystems). Each sample was measured in quadruplicate; data are shown as mean values $\pm$ SEM.

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2.2 $p57^{Kip2}$ regulates glial fate decision in adult neural stem cells

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Abstract

Our recent studies revealed $p57^{Kip2}$ as an intrinsic regulator of late gliogenesis and demonstrated that in oligodendroglial precursor cells $p57^{Kip2}$ inhibition leads to accelerated maturation. Adult neural stem cells have been described as a source of glial progenitors; however, the underlying mechanisms of cell fate specification are still poorly understood. Here, we have investigated whether $p57^{Kip2}$ can influence early events of glial determination and differentiation. We found that Sox2/GFAP double-positive cells express $p57^{Kip2}$ in stem cell niches of the adult brain. Short-hairpin RNA-mediated suppression of $p57^{Kip2}$ in cultured adult neural stem cells was found to strongly reduce astroglial characteristics, while oligodendroglial precursor features were increased. Importantly, this anti-astrogenic effect of $p57^{Kip2}$ suppression dominated the bone morphogenetic protein-mediated promotion of astroglial differentiation. Moreover, we observed that in $p57^{Kip2}$ knockdown cells, the BMP antagonist chordin was induced. Finally, when $p57^{Kip2}$-suppressed stem cells were transplanted into the adult spinal cord, fewer GFAP-positive cells were generated and oligodendroglial markers were induced when compared with control cells, demonstrating an effect of *in vivo* relevance.

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Contribution on experimental design, realization and publication

Experimental design and practical realization were accomplished by Janusz Joachim Jadasz. Figure 1 was accomplished by FJR. Transplantation of suppressed adult neural stem cells were accomplished in assistance by Janusz Joachim Jadasz. Janusz Joachim Jadasz, FJR together with PK were involved in writing the manuscript including preparation of all figures. The manuscript was subsequently reviewed, amended and approved by all co-authors.

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3306 **RESEARCH ARTICLE** **DEVELOPMENT AND STEM CELLS**

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p57kip2 regulates glial fate decision in adult neural stem cells

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SUMMARY

Our recent studies revealed p57kip2 as an intrinsic regulator of late gliogenesis and demonstrated that in oligodendroglial precursor cells p57kip2 inhibition leads to accelerated maturation. Adult neural stem cells have been described as a source of glial progenitors; however, the underlying mechanisms of cell fate specification are still poorly understood. Here, we have investigated whether p57kip2 can influence early events of glial determination and differentiation. We found that Sox2/GFAP double-positive cells express p57kip2 in stem cell niches of the adult brain. Short-hairpin RNA-mediated suppression of p57kip2 in cultured adult neural stem cells was found to strongly reduce astroglial characteristics, while oligodendroglial precursor features were increased. Importantly, this anti-astrogenic effect of p57kip2 suppression dominated the bone morphogenetic protein-mediated promotion of astroglial differentiation. Moreover, we observed that in p57kip2 knockdown cells, the BMP antagonist chordin was induced. Finally, when p57kip2-suppressed stem cells were transplanted into the adult spinal cord, fewer GFAP-positive cells were generated and oligodendroglial markers were induced when compared with control cells, demonstrating an effect of in vivo relevance.

KEY WORDS: Astrocyte fate, Oligodendroglial differentiation, Multiple sclerosis, Regeneration, Spinal cord injury, Rat, Cdkn1c

INTRODUCTION

Adult neurogenesis mainly occurs in two regions of the central nervous system (CNS), the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Alvarez-Buylla et al., 2001). Both regions contain adult neural stem cells (NSCs), which have the capacity to self-renew and differentiate into neurons and glia (Gritti et al., 1999; Kriegstein and Alvarez-Buylla, 2009). NSCs and progenitor cells are embedded in a cellular and extracellular micro-environment, the so-called stem cell niche (Palmer et al., 2000; Doetsch, 2003). SVZ-derived NSCs are able to generate neuronal-determined progenitors and tangentially migrate along the rostral migratory stream (RMS) to the olfactory bulb to be incorporated as interneurons (Doetsch and Alvarez-Buylla, 1996). Furthermore, it has been demonstrated that glial fibrillary acidic protein (GFAP)-expressing radial glia astrocytes of the SVZ also possess stem cell properties (Doetsch et al., 1999; Laywell et al., 2000). NSCs from the SGZ divide along the hilus and generate a continuous pool of differentiating neuronal precursors, which functionally integrate into the granular hippocampal cell layer (Kuhn et al., 1996; van Praag et al., 2002). However, these hippocampal-derived NSCs are not restricted to generate only neurons but they can also give rise to oligodendroglial cells in culture (Rivera et al., 2006) as well as in vivo (Jessberger et al., 2008). Of note, under inflammatory conditions, SVZ NSCs are also able to generate oligodendroglial cells (Picard-Riera et al., 2002; Menn et al., 2006; Nait-Oumesmar et al., 2007). In addition, it has been shown that NSCs from the subcallosal zone, a caudal extension of the SVZ, migrate into the corpus callosum to become oligodendrocytes (Seri et al., 2006).

The CNS has a limited capacity for regeneration, which is why traumatic injuries, demyelinating or degenerative diseases generally result in irreversible deficits. In the pathology of multiple sclerosis (MS), an immune-driven inflammatory disease featuring myelin loss, axonal degeneration as well as astrogliosis (Trapp et al., 1999; Bitsch et al., 2000), transient phases of recovery occur owing to remyelination (Franklin and Ffrench-Constant, 2008). Functional cell replacement is a consequence of oligodendrocyte precursor cell (OPC) or NSC activation and differentiation (Chang et al., 2000; Picard-Riera et al., 2002; Menn et al., 2006; Nait-Oumesmar et al., 2007; Rivera et al., 2010). Despite its spontaneous character, the overall remyelination efficiency remains low and is further decreased with disease progression, which is thought to be due to the presence of inhibitory factors (Scolding et al., 1998; Wolswijk, 1998; Chang et al., 2000; Chang et al., 2002; Kuhlmann et al., 2008; Kremer et al., 2009; Kremer et al., 2011). Identification of such inhibitory components and knowledge about signal transduction pathways to interfere with thus allow development of strategies to promote cell replacement and endogenous remyelination.

We have investigated inhibitory processes of myelinating glial cell differentiation and found that the p57kip2 protein (Cdkn1) efficiently blocks both peripheral Schwann cell maturation as well as OPC differentiation (Küry et al., 2002; Heinen et al., 2008; Kremer et al., 2009). Upon long-term suppression, a number of differentiation associated processes (such as cell-cycle exit, morphological maturation, gene expression and myelin production) were affected and maturation was promoted.

In view of its strong inhibitory effect on progenitor cells we wondered whether glial fate determination as an early differentiation step might also be under the control of p57kip2. We therefore investigated its expression among stem cells in adult CNS niches and studied the consequences of decreased p57kip2 expression levels in cultured and transplanted NSCs. This revealed

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a strong anti-astrogenic effect, generation of oligodendroglial features as well as induction of BMP antagonists upon p57kip2 suppression in cultured NSCs. Modulation of p57kip2 expression or activity could therefore be used to promote myelinating glial cell replacement and to limit the degree of astrogliosis at the same time.

MATERIALS AND METHODS

Animal subjects

Adult female Wistar rats with a weight of 160-180 g (3-4 months old) were used as donors for the NSC isolation and for immunohistochemical analysis of stem cell niches. Adult female Fischer 344 rats were used for transplantation experiments. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines.

NSC preparation

Adult rats were anesthetized using ISOFLURAN (DeltaSelect, Langenfeld, Germany) and killed by decapitation. Brains and spinal cords were removed and put in 4°C phosphate-buffered saline (PBS; PAA Laboratories, Pasching, Austria). Overlying meninges and blood vessels were removed. Hippocampus (HC) and ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle (SVZ), were aseptically removed and dissociated mechanically. The cell suspension was washed in PBS and further digested in PPD solution containing papain (0.01%, Worthington Biochemicals, Lakewood, USA), 0.1% dispase II (Boehringer, Ingelheim, Germany), DNase I (0.01%, Worthington Biochemicals) and 12.4 mM $MgSO_4$, dissolved in HBSS (PAA Laboratories) for 30 minutes at 37°C. The cell suspension was triturated every 10 minutes until the tissue was completely digested, spun down and washed in Neurobasal (NB) medium (Gibco BRL, Karlsruhe, Germany) supplemented with B27 (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin/0.1 mg/l streptomycin (PAN Biotech, Aidenbach, Germany). Cells were resuspended in NB medium supplemented with 2 μg/ml heparin (Sigma-Aldrich, Taufkirchen, Germany), 20 ng/ml FGF-2 (R&D Systems, Wiesbaden-Nordenstadt, Germany) and 20 ng/ml EGF (R&D Systems). Cultures were maintained as neurospheres in uncoated culture flasks at 37°C in a humidified incubator with 5% CO_2. Single cells began to form spheres within 5-7 days and continued to grow during the following weeks. Half of the medium was changed every 3 days and cell cultures were passaged weekly by means of limited accutase (PAA Laboratories) digestion at 37°C for 10 minutes and subsequent trituration. A total of 5×10^4 cells/ml were seeded in T75 culture flasks in fresh growth medium. In order to allow differentiation to take place, dispersed cells were plated on poly-L-ornithine/laminin (100 μg/ml and 5 μg/ml, Sigma-Aldrich)-coated cell culture dishes or glass cover slips and incubated in control culture medium [α-MEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA)] in absence or presence of 10 ng/ml BMP2 and 10 ng/ml BMP4 (R&D Systems).

Stem cell transfection and immunocytological procedures

Neurosphere cultures from passage numbers three to six were used to generate dispersed NSCs. Transfection with shRNA-mediated suppression constructs [H1: pSUPER empty control vector and H1-kip2: p57kip2_1 suppression vector (Heinen et al., 2008)] were carried out in combination with a citrine expression vector (Heinen et al., 2008) using a Lonza nucleofection device (program A-033 high efficiency) and the adult rat NSC nucleofector kit (Lonza, Basel, Switzerland). Following this procedure, ~30-50% of surviving cells were transfected. For marker expression analysis, NSCs were fixed with 4% paraformaldehyde/PBS solution, PBS washed, blocked for 2 hours using 10% normal goat serum in PBS and subjected to antibody incubation at 4°C overnight: rabbit anti-p57kip2 (1:200, Sigma-Aldrich), rabbit anti-caspase3 (1:500, Cell Signaling Technology, Leiden, The Netherlands), rabbit anti-Ki67 (1:500, Millipore, Schwalbach, Germany), rabbit anti-GFAP (1:1000, Dako, Hamburg, Germany), mouse anti-nestin, (1:300, Millipore), mouse anti-GFAP (1:1000, Millipore), rabbit anti-AQP4 (1:500, Sigma-Aldrich), mouse anti-GalC (1:350, Millipore), mouse anti-O4 (1:1000, Millipore) and rabbit anti-GST-π (1:1000, Enzo). Following PBS washes secondary anti-mouse and anti-rabbit antibodies conjugated with either Alexa Fluor594, Alexa Fluor350 fluorescent dyes (1:1000, Invitrogen), Cy3 or FITC (1:500, Millipore) were added for 2 hours at room temperature. Cells were mounted under Citifluor (Citifluor, Leicester, UK) and analyzed using an Axio Cam HRc microscope (Zeiss, Jena, Germany) or for confocal images with a Zeiss LSM 510 Axiovert 200 M microscope (Zeiss, Jena, Germany). Citrine-positive cells on nine different fields per glass coverslip were counted and analyzed for marker expression. For live cell imaging, adult neural stem cells were photographed every 8 hours starting at time point 24 hours post-transfection lasting up to 192 hours.

For preparation of cryosections, neurospheres were allowed to settle for 20 minutes at room temperature and then fixed with 4% PFA for 45 minutes at room temperature. Cryoprotection was carried out in 30% sucrose for 5 hours at 4°C. Embedded in Tissue Tek (Sakura Finetek Europe, Zoesterwoude, The Netherlands), neurospheres were cut into 14 μm sections and processed for immunohistochemistry. Sections were blocked using 1% normal goat serum with 0.1% Triton in PBS for 1 hour at room temperature and subjected to antibody incubation (mouse anti-nestin, 1:300, Millipore; mouse anti-GFAP, 1:1000, Millipore; rabbit anti-p57kip2, 1:200, Sigma-Aldrich) at 4°C overnight in antibody incubation solution (1% normal goat serum with 0.03% Triton in PBS). Staining with secondary antibodies was performed as described above. Data are shown as mean values±s.e.m. and *t*-test was applied in order to determine statistical significance and was performed with Prism 5.0 (GraphPad Software).

RNA preparation, cDNA synthesis and quantitative reverse transcription (RT)-PCR

Transfected NSCs were isolated after 24 hours using fluorescence-activated cells sorting (FACS) by means of citrine co-expression (FACSaria; BD, Franklin Lakes, NJ, USA) and then cultured for up to 7 days. Total RNA purification from cells was carried out using the RNeasy procedure (Qiagen, Hilden, Germany). Isolated RNA was reverse transcribed using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative determination of gene expression levels was performed on a 7900HT sequence detection system (Applied Biosystems) using Power SybrGreen universal master mix (Applied Biosystems). Primer sequences were determined using PrimerExpress 2.0 software (Applied Biosystems) and tested for the generation of specific amplicons: GFAP_fwd, CTGGTGTGGAGTGCCTTCGT; GFAP_rev, CACCAACCAGCTTCCGAGAG; CGT_fwd, CCGGCCACCCT-GTCAAT; CGT_rev, CAGGGAGACGAGTCACAACGT; EZH2_fwd, GGGCTGCACACTGCAGAAA; EZH2_rev, CATGGTTAGAGG-AGCCGTCC; p57kip2_fwd, CAGGACGAGAATCAGGAGCTGA; p57kip2_rev, TTGGCGAAGAAGTCGTTCG; chordin_fwd, ACTAGCTCACGTCCCCTTGAAG; chordin_rev, GGCCTGGAGC-TCTCGAAGTA; noggin_fwd, CTGGTGGACCTCATCGAACA; noggin_rev, GCGTCTCGTTCAGATCCTTCTC; GAPDH_fwd, GAACGGGAAGCTCACTGGC; GAPDH_rev, GCATGTCAGA-TCCACAACGG; ODC_fwd, GGTTCCAGAGGCCAAACATC; ODC_rev, GTTGCCACATTGACCGTGAC. GAPDH and ODC were used as reference genes, and relative gene expression levels were determined according to the ΔΔCt method (Applied Biosystems). Each sample was measured in quadruplicate; data are shown as mean values±s.e.m. and *t*-test was applied in order to determine statistical significance (Prism 5.0; GraphPad Software).

Cell transplantation into the intact spinal cord

Animals were anesthetized with a cocktail of ketamine (62.5 mg/kg; WDT, Garbsen, Germany), xylazine (3.175 mg/kg; WDT, Garbsen, Germany) and acepromazine (0.625 mg/kg, Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline solution. Prior to NSC implantation, animals received a partial laminectomy at cervical level C3. Then a total volume of 2 μl cell suspension containing either 1.2×10^5 control NSC/μl (H1 and citrine vector co-transfected; n=6) or 1.2×10^5 p57kip2 suppressed NSC/μl (H1-kip2 and citrine vector co-transfected; n=6) was injected under stereotactical guidance (mediolateral midline, dorsoventral 0.8 mm) into the dorsal spinal cord through a pulled glass micropipette (100 μm internal

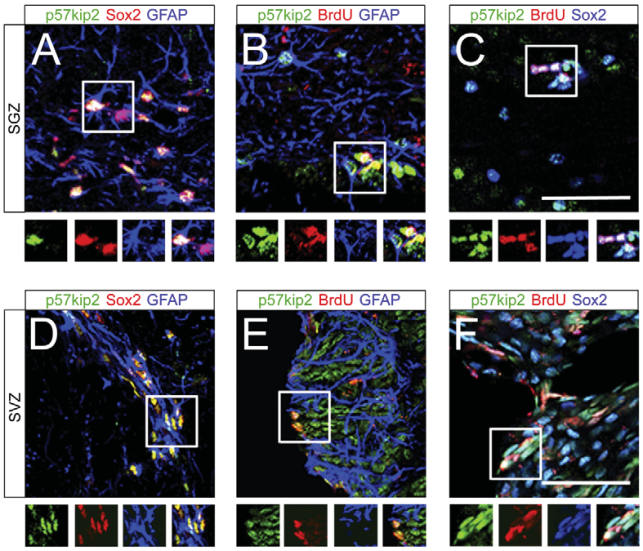

Fig. 1. p57kip2 is expressed in adult brain stem cell niches. (**A-F**) Immunohistofluorescent stainings revealed overlapping p57kip2, Sox2, GFAP and BrdU signals in SGZ (A-C) and SVZ (D-F) niches, demonstrating p57kip2 expression in stem cells in vivo. All cells are shown as single and merged pictures. Scale bars: 50 μm.

diameter) using a Picospritzer Π device (General Valve, Fairfield, USA). Air-driven pulses of 15 nl per pulse, 20 pulses per site, were delivered. The micropipette tip remained in place for 25 seconds before withdrawal. The implantation site was covered with gelfoam (Gelita Tampon; Braun, Germany) before readapting muscular layers and stapling the skin above the lesion. Four days post-operation, animals were transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cords were removed, post-fixed overnight and cryoprotected in 30% sucrose, cut into sagittal 35 μm cryostat sections and processed for immunohistochemistry. Immunofluorescence stainings were performed on free-floating sections to assess NSC differentiation parameters using primary antibodies at 4°C overnight: goat anti-GFP/citrine (1:500; Biotrend, Cologne, Germany), rabbit anti-GFAP (1:1000, Dako), rabbit anti-GST-π (1:2000, Enzo) and mouse anti-adenomatous polyposis coli for oligodendrocytes (APC; CC1; 1:500, Calbiochem, Darmstadt, Germany). Sections were incubated with rhodamine-X- (1:500, Dianova, Hamburg, Germany), Alexa Fluor488- (1:1000, Molecular Probes) or Cy5- (1:500, Dianova) conjugated donkey secondary antibodies for 2 hours at room temperature. Sections were mounted onto glass slides and covered with Prolong Antifade (Invitrogen GmbH, Karlsruhe, Germany). Analysis was performed by confocal scanning laser microscopy (Leica TCS-NT, Wetzlar, Germany). Colocalization of citrine-labeled NSCs with differentiation markers was determined by analyzing between 30-35 optical sections through the *z*-axis of the coronal section at 400× magnification. Colocalization was confirmed once the differentiation marker was spatially associated with the citrine signal through subsequent optical sections in the *z*-axis. Expression sites were analyzed with two sections per marker and by analyzing one field of view in three different regions: (1) directly at the injection site in the white matter (ISWM); (2) caudal to the injection site in the white matter (WM); and (3) above the injection site in the grey matter (GM). Data are shown as mean values±s.e.m. and *t*-test was applied in order to determine statistical significance (Prism 5.0; GraphPad Software).

BrdU labeling and immunohistochemical staining procedures

In vivo labeling of dividing cells was performed by intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich) at 50 mg/kg of body weight using a sterile solution of 10 mg/ml BrdU dissolved in 0.9% (w/v) NaCl solution. Injections were performed daily on 4 consecutive days (day 1 to 4). At day 30, rats were anesthetized and sacrificed by transcardial perfusion. Brains were removed, post-fixed in paraformaldehyde overnight at 4°C, cryoprotected in 30% (w/v) sucrose, 0.1 M sodium phosphate solution (pH 7.4) and cut into 40 μm sagittal sections using a sliding microtome on dry ice. Free-floating sections were treated with 0.6% H_2O_2 in Tris-buffered saline [TBS; 0.15 M NaCl, 0.1 M Tris-HCl (pH 7.5)] for 30 minutes. For BrdU detection, tissue pre-treatment was performed as described previously (Couillard-Despres et al., 2005). Sections were blocked [TBS, 0.1% Triton X-100, 1% bovine serum albumin, 0.2% teleostean gelatine (Sigma, Taufkirchen, Germany)] and primary antibodies were applied overnight at 4°C: rat anti-BrdU (1:500, Oxford Biotechnology, Oxford, UK), rabbit anti-GFAP (1:1000, Dako), guinea pig anti-GFAP (1:500, Progen, Germany), goat anti-Sox2 (1:500, Santa Cruz Laboratories, Santa Cruz, USA), rabbit anti-p57kip2 (1:400, Sigma-Aldrich). Sections were washed and incubated with fluorochrome-conjugated secondary antibodies overnight at 4°C: donkey anti-goat, -mouse, -rabbit or -rat conjugated with Alexa Fluor488 (1:1000, Molecular Probes, Eugene, USA), rhodamine X (1:500, Dianova, Hamburg, Germany), Cy5 or biotin (1:500, Jackson ImmunoResearch, West Grove, USA). Sections were put onto slides and mounted under Prolong Antifade kit (Molecular Probes). Photodocumentation and analysis were performed by confocal scanning laser microscopy.

RESULTS

p57kip2 is expressed by adult NSCs of the two neurogenic niches

Immunohistochemical analysis revealed that the p57kip2 protein is expressed in the neural stem cell niches SGZ and SVZ (Fig. 1). Sox2/GFAP-expressing cells have been proposed to be resident NSCs within these brain regions (Graham et al., 2003) and we found that Sox2/GFAP double-positive cells also expressed p57kip2 in SGZ and SVZ (Fig. 1A,D). NSCs are slow proliferating cells with a self-renewal capacity, which is typically identified by their label-retaining potential. Thus, we examined the expression of p57kip2 in GFAP- and Sox2-expressing cells that had retained 5-bromo-2-deoxyuridine (BrdU) 26 days after a daily injection of BrdU for 4 consecutive days. This revealed in both niches a large number of BrdU label-retaining GFAP-expressing cells that were also p57kip2 positive (Fig. 1B,E), as well as BrdU-positive Sox2/p57kip2 co-expressing cells (Fig. 1C,F). Of note, no colocalization of p57kip2 with NeuN was detected, suggesting that p57kip2 is absent in mature DG neurons (data not shown).

p57kip2 suppression in cultured adult neural stem cells

Following the demonstration of p57kip2-positive cells in stem cell niches of the adult brain, we tested whether neurosphere cells express p57kip2 and whether colocalization with stem/progenitor cell markers such as GFAP and nestin could be observed in vitro. This revealed that all neurosphere cells were p57kip2 positive (Fig. 2A) and that expression was maintained in dispersed and adherent cells (data not shown). We next addressed the role of p57kip2 in stem cell differentiation and suppressed its expression using short-hairpin RNAs (shRNAs). This procedure has already successfully been applied to glial progenitor cells and was found to be specific for p57kip2 without inducing a non-specific defense reaction, the so-called interferon response (Heinen et al., 2008; Kremer et al., 2009). Dispersed adult SGZ and SVZ-derived neurosphere cells were transfected with suppression and control constructs in presence of a citrine-expressing vector, FACS isolated after 24 hours for gene expression measurements and plated on laminin-coated surfaces in differentiation permissive medium (α-MEM) (Rivera et al., 2006). Real-time quantitative RT-PCR confirmed the knockdown of p57kip2 transcription (Fig. 2B) and anti-p57kip2 staining showed that p57kip2 protein levels were also strongly reduced in suppressed but not in control transfected cells (identified by means of citrine expression; Fig. 2C-D′). To elucidate whether the gene suppression affects apoptotic cell death or proliferation, we performed anti-caspase3 and anti-Ki67 stainings, respectively (Fig. 2E,F). This revealed a significantly reduced cell death rate upon p57kip2 suppression while the proliferation rate of differentiating stem cells was slightly increased. Note that, under the conditions applied, the majority of cultured cells were not proliferating. In general, when cell specific markers were evaluated using immunocytofluorescent stainings, only transfected cells (visualized by means of citrine co-expression) were scored.

Interestingly, we observed different cellular morphologies between p57kip2 suppressed and control-transfected cells (for both populations SGZ and SVZ) and therefore quantitatively analyzed the distribution of cells adopting astroglial, oligodendroglial or other (neuronal) morphologies (7 days post transfection). Our analyses revealed that the majority of control-transfected NSCs (termed H1 in Fig. 2G,H,H′) adopted a flat astrocyte type I-like or multipolar astrocyte type II-like morphology without secondary processes, whereas most of the p57kip2-suppressed NSCs acquired a multipolar shape with secondary processes resembling an oligodendroglial-like morphology (H1-kip2 in Fig. 2G,I). We detected eight times more oligodendroglial-like phenotypes in p57kip2-suppressed NSCs than in control-transfected cells, whereas the number of cells with a non-glial shape was not affected (Fig. 2G,J). In order to study single cells upon modulation of p57kip2 expression, we performed live cell imaging and followed control-transfected (H1) and p57kip2-suppressed (H1-kip2) cells during a period from day 1 to 8 (hours 24 to 192) in control medium (α-MEM), as well as following stimulation with bone morphogenetic proteins 2 and 4 (BMP2/4) (Gross et al., 1996). We conducted two independent experiments and observed in total 284 H1 transfected and 224 H1-kip2 transfected single cells during the whole period (24 to 192 hours). We discriminated between cells with oligodendroglial morphologies and flat cells with astrocytic appearance. This analysis revealed that in both media p57kip2 knockdown promotes oligodendroglial maturation. In α-MEM we found a shift from 24.47±3.94% (H1) to 67.44±1.61% (H1-kip2) of cells with oligodendroglial appearance (*t*-test, P=0.0097). In the presence of BMPs, we detected a shift from 25.86±1.86% (H1) to 63.76±1.95% (H1-kip2) of cells with oligodendroglial appearance (*t*-test, P=0.0050). This is shown for a representative p57kip2-suppressed (H1-kip2) stem cell developing an oligodendroglial morphology (Fig. 3B) and a representative control-transfected (H1) stem cell adopting a flat astrocytic morphology (Fig. 3A). Morphological changes were first observed after ~72 hours and that

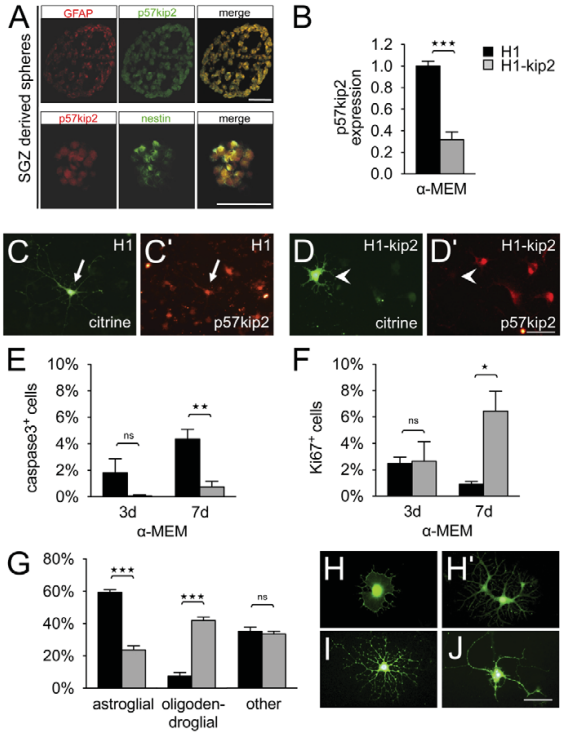

Fig. 2. Characterization of neurospheres prepared from the SGZ of the adult hippocampal dentate gyrus. (**A**) Confocal images of cryosectioned neurospheres stained for p57kip2 and stem/progenitor cell markers revealed co-expression with GFAP and nestin. (**B**) Adult neural SGZ neurospheres were dissociated and dispersed cells were transfected with gene suppression constructs in order to mediate downregulation of p57kip2. Quantitative RT-PCR analysis of sorted control transfected (H1) and p57kip2 suppressed (H1-kip2) cultured NSCs demonstrated that p57kip2 transcript levels were significantly lowered. (**C-D′**) Anti-p57kip2 immunostaining 3 days after NSC transfection (visualized by means of citrine expression; arrow marks an expressing cell, arrowhead marks absent expression), showed that H1-kip2 transfected NSCs were devoid of p57kip2 protein. (**E**,**F**) Anti-caspase3 and anti-Ki67 immunostainings showed that 3 and 7 days after p57kip2 suppression, apoptotic cell death was reduced and that proliferation rate was slightly increased after 7 days. Suppression of p57kip2 induces morphological alterations. (**G**) Determination of relative cell numbers with astroglial, oligodendroglial and other morphologies in control transfected (H1) and p57kip2-suppressed (H1-kip2) NSCs derived from the SVZ (similar results observed in SGZ cells, data not shown). (**H-J**) Representative H1 transfected cells with astroglial-like (H,H′), non glial- (J) and an H1-kip2 transfected cell with oligodendroglial-like (I) features. Data are mean±s.e.m. derived from n=3 (caspase3, Ki67) and n=6 (morphology) independent experiments. *t*-test (***P<0.001; **P<0.01; *P<0.05; ns, not significant). Scale bars: 50 μm.

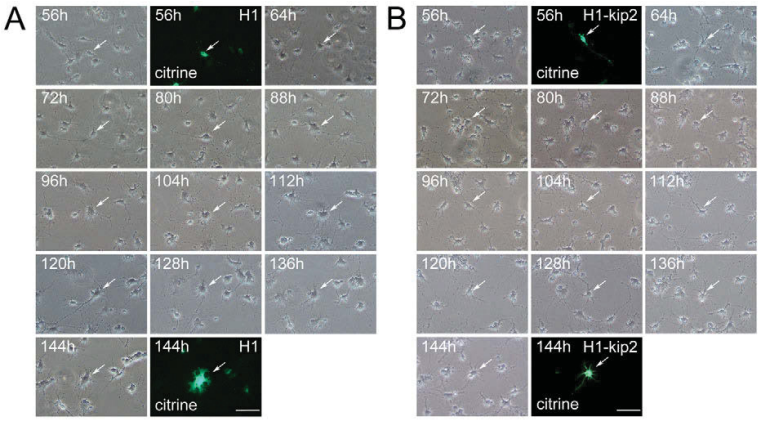

Fig. 3. Live cell imaging. (**A**,**B**) A representative SGZ-derived stem cell transfected with the empty control vector (H1) (A) and a representative SGZ-derived stem cell transfected with the p57kip2 suppression vector (H1-kip2) (B) observed during the time window 56 to 144 hours post-transfection (cultured in α-MEM). After 72 hours, morphological changes become apparent and the p57kip2-suppressed cell develops an oligodendroglial morphology, whereas the control transfected cell adopts a flat astrocytic morphology. No signs of selective proliferation or cell death could be observed. Arrows mark transfected and citrine-labeled cells. Scale bars: 50 μm.

in this analysis no dividing (neither H1- nor H1-kip2 transfected) cells were observed. This is in contrast to the slight proliferation differences shown in Fig. 2F and might be due to the much lower cell number that has been studied when compared with the Ki67 expression analysis. In addition, only transfected cells that we were able to follow during the whole observation period were finally scored for morphological changes, as we wanted to reveal when and to what extent differentiation occurs.

Astrocyte differentiation is downregulated upon p57kip2 suppression

We next examined whether the expression of glial lineage markers was dependent on p57kip2 expression levels. We observed a strong reduction of GFAP transcript levels in p57kip2-suppressed cells compared with control transfected cells derived from the SGZ (Fig. 4A). This downregulation also resulted in a significant decrease of the percentage of GFAP-positive cells (Fig. 4B), both under standard conditions as well as under dominant astrocyte-promoting conditions such as the stimulation with BMP2/4 (Gross et al., 1996). GFAP-negative p57kip2-suppressed NSCs displayed oligodendroglial-like morphologies, whereas GFAP-expressing control cells strongly resembled astrocytes (Fig. 4C-C″,D-D″). Three different p57kip2 suppression constructs were used, all of which resulted in decreased GFAP expression levels. For reasons of simplicity, only data of experiments carried out with the first construct (termed H1-kip2) (Heinen et al., 2008) are shown. However, p57kip2 overexpression (Heinen et al., 2008) substantially increased the percentage of GFAP-positive cells, thus enhancing astrogenesis (Fig. 4E). H1-kip2 transfected SVZ-derived stem cells downregulated GFAP to the same degree (data not shown) and were furthermore shown to turn down aquaporin 4 (AQP4) expression upon p57kip2 knockdown in control medium, as well as in presence of BMPs (Fig. 4F,G-H″). AQP4 encodes an astroglial end-feet marker and we found that under the conditions applied some SVZ, but no SGZ-derived stem cells expressed it during the differentiation process.

p57kip2 suppression leads to an increase of OPC markers

Since astrogenic features were revealed to be inhibited upon p57kip2 suppression, we determined whether this gene knockdown also affected oligodendroglial parameters. p57kip2-suppressed SGZ cells displayed raised transcript levels of the polycomb group protein EZH2 (Fig. 5A), which was shown to be expressed at high levels in NSCs that differentiate into OPCs (Sher et al., 2008). In addition, the OPC marker ceramide galactosyl transferase (CGT) was upregulated in p57kip2-suppressed cells (Fig. 5B) and we observed a significant increase in the percentage of galactocerebroside-positive (GalC) cells after p57kip2 knockdown (Fig. 5C,D-E″). Interestingly, in presence of BMP2/4, this induction process was even more emphasized. Despite the fact that almost 30% of the p57kip2-suppressed cells differentiated into GalC-expressing cells in presence of BMP2/4 (Fig. 5C), we could not detect differences in the expression of mature oligodendrocyte markers such as myelin basic protein (MBP) or 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; data not shown). In order to reinforce these results, we evaluated the effect of p57kip2 suppression in SVZ-derived NSCs. We found that, in these cells, the expression of O4, another early oligodendroglial marker, was elevated upon reduction of p57kip2 expression (Fig. 5F,H-I″) as was the expression of GST-π (Fig. 5G). Note that GST-π positivity was also significantly elevated in p57kip2-suppressed SGZ-derived NSCs (data not shown). This suggests that the p57kip2 suppression induces an OPC stage and that additional signals are necessary to further promote the generation of mature oligodendrocytes. To find out whether selection (i.e. increased proliferation or promoted survival) accounts for the observed effects, we performed anti-Ki67 and anti-caspase3 staining in combination with GalC, O4, GFAP and AQP4 antibodies at two different time points (3 days and 7 days) after transfection of SGZ and SVZ cells. This analysis revealed that, at both time points, none of the O4- or GalC-positive cells were Ki67 or caspase3 labeled (data not shown), indicating that the generation of these cells is most likely not due to an increased proliferative activity or enhanced survival. Given that suppression of p57kip2 exerts an overall cell protective effect (Fig. 2E), we can also exclude that the strong difference among GFAP-positive cells results from an increased death in response to p57kip2 suppression. This was also confirmed using GFAP/caspase3 double stainings (data not shown). In addition, we observed that the total cell numbers between the two conditions (control and p57kip2-suppressed cells) were quite stable. We therefore conclude that p57kip2 can regulate cellular fate switching.

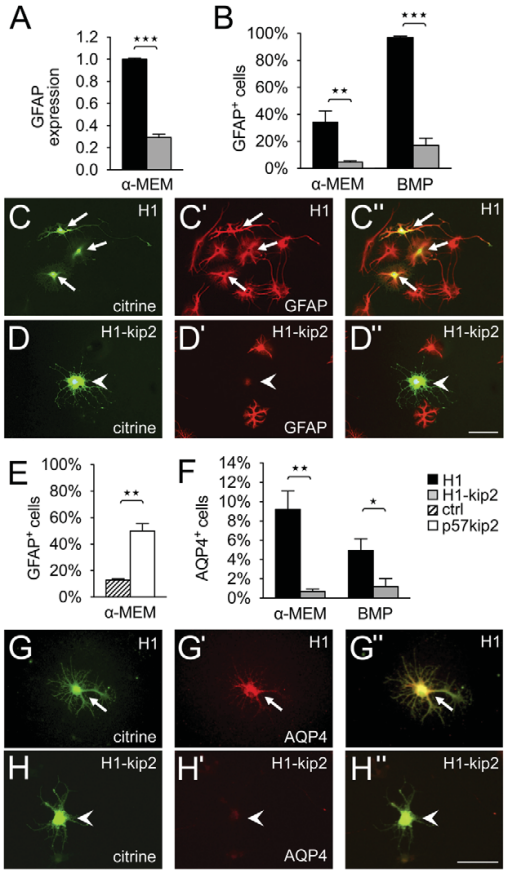

Fig. 4. Downregulation of astrocyte markers following p57kip2 suppression. (**A-D″**) GFAP downregulation, as revealed by transcript levels in sorted (FACS) cells (A) as well as by immunostaining of control (H1) and p57kip2-suppressed (H1-kip2) SGZ cells 7 days post-transfection (B-D″). (**E**) Induced GFAP expression by p57kip2 overexpression. (**F-H″**) AQP4 protein downregulation in SVZ cells upon p57kip2 knockdown. Arrows mark expressing cells; arrowheads mark absent expression. Data are mean±s.e.m. derived from *n*=6 (GFAP) and *n*=3 (AQP4) independent experiments. *t*-test (****P*<0.001; ***P*<0.01; **P*<0.05; ns, not significant). Scale bars: 50 µm. α-MEM, control medium; BMP, BMP2/4-containing astrocyte-inducing medium.

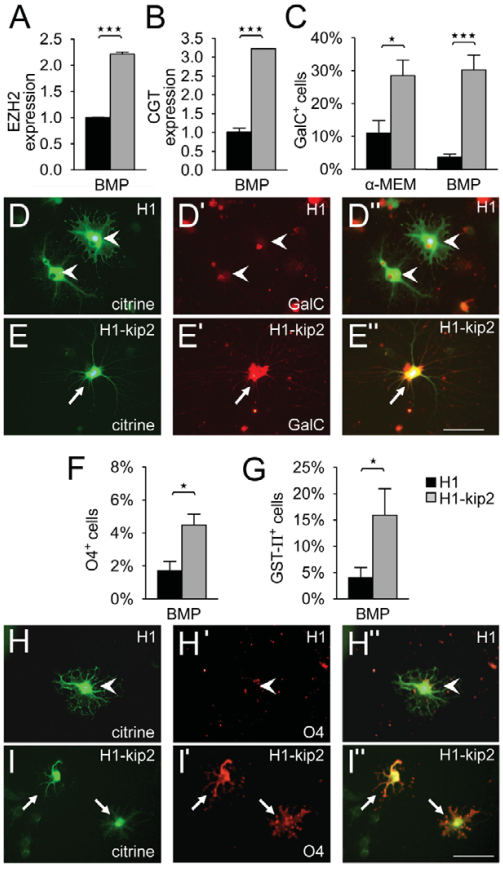

Fig. 5. Upregulation of oligodendroglial precursor markers following p57kip2 suppression. Adult SGZ neural stem cells were sorted (FACS via citrine co-expression) after p57kip2 knockdown and plated on laminin-coated coverslips for 7 days in control (α-MEM) or astrocyte-promoting (BMP) conditions. (**A**,**B**) Increased EZH2 (A) and CGT (B) transcript levels were observed in sorted and p57kip2-suppressed NSCs. (**C-E″**) In addition, an increased percentage of GalC-positive SGZ cells upon p57kip2 knockdown was observed. (**F**,**H-I″**) Induction of O4 expression in p57kip2-suppressed SVZ cells. Arrows mark expressing cells, arrowheads mark absent expression. (**G**) Induction of GST-π positivity in p57kip2-suppressed SVZ cells. Data are mean±s.e.m. derived from *n*=6 (EZH2, CGT, GalC, O4, GST-π) independent experiments. *t*-test (****P*<0.001; **P*<0.05). Scale bars: 50 µm. α-MEM, control medium; BMP, BMP2/4 containing astrocyte-inducing medium.

Evaluation of neuronal and stem cell marker expression

In order to find out whether p57kip2 levels also affect neurogenesis, we performed anti-β-tubulin, anti-Map2ab and anti-DCX staining on transfected SVZ and SGZ cells. In SVZ cells, the number of β-tubulin-positive cells was downregulated upon p57kip2 suppression, whereas SGZ cells showed no β-tubulin reaction. When compared with the GFAP downregulation, the reduction of β-tubulin-positive cells was small (12.08±1.4% to 6.28±3.3% from control to p57kip2 suppressed cells; mean±s.e.m.; *n*=4). Both cell types non-significantly downregulated levels of Map2ab-positive cells (6.46±1.78% to 4.67±0.77%; mean±s.e.m.; *n*=3). In SGZ cells, the amount of DCX positivity was also slightly lowered (29.03±1.19% to 25±1.75% from control to p57kip2-suppressed cells; mean±s.e.m.; *n*=3). We conclude that a mild reduction of neuronal marker expression accompanies the strong reduction of astrocyte markers.

Of note, interesting observations resulted when we performed anti-nestin stainings. As a consequence of the p57kip2 knockdown, the number of nestin/GFAP double-positive cells was reduced, but the number of nestin^{+}/GFAP^{-} cells was increased. However, the overall number of nestin-positive cells remained unchanged (this

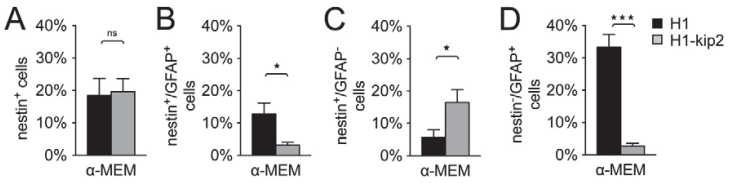

Fig. 6. p57kip2-suppressed SVZ-derived NSCs show no difference in the expression of the stemness marker nestin. (**A-D**) Immunofluorescent determination shows no significant change in the number of nestin-positive cells between control transfected and p57kip2-suppressed cells 7 days post-transfection (A), whereas the number of nestin/GFAP double-positive cells was decreased (B), the number of nestin-positive/GFAP-negative cells was increased (C) and the number of nestin-negative/GFAP-positive was strongly decreased (D). Data are mean±s.e.m. derived from *n*=9 independent experiments. *t*-test (**P*<0.05; ****P*<0.001; ns, not significant). α-MEM, control medium.

applies for SGZ and SVZ cells and is shown for SVZ cells in Fig. 6A-D). This indicates that the expression of nestin as a stem cell marker is not dependent on p57kip2.

Transplantation of p57kip2 suppressed stem cells

In order to reveal whether these observations are of in vivo relevance and whether the previously observed tendency to generate astrocytes from transplanted stem cells (Setoguchi et al., 2004) can be counteracted by means of modulated p57kip2 expression, we injected transfected SGZ-derived NSCs into the dorsal spinal cord of healthy rats. Owing to the transient nature of our genetic modulation of stem cells, spinal cords were removed at an early time point (4 days following transplantation) and GFAP, GST-π and CC1 expression on citrine-labeled stem cells was investigated. Similar to the studies on cultured stem cells, empty vector transfected non-suppressed cells were used as controls. Consistent with our previous in vitro observations, significantly fewer transplanted NSCs expressed GFAP protein when p57kip2 was suppressed prior to transplantation. These modulated cells showed a significant induction of the oligodendroglial precursor marker GST-π (Rivera et al., 2009) and tended to induce expression of the oligodendroglial marker CC1 [Fig. 7A; representative pictures of injection site in the white matter (ISWM) shown in Fig. 7B-E″]. This indicated that lowered p57kip2 levels influence the capacity of transplanted cells to react to astrogenic signals as they occur in the dorsal spinal cord (Fuller et al., 2007) and to induce OPC features instead.

BMP antagonists are upregulated upon p57kip2 suppression

To elucidate the underlying mode of action, we investigated levels of gene expression of major BMP antagonists such as chordin, noggin and follistatin (Jablonska et al., 2010; Walsh et al., 2010). Consistent with our findings that upon p57kip2 suppression astrocytic markers were downregulated, and that these cells lost astrocytic morphological features, we detected a strong induction of chordin expression in FACS isolated cells (Fig. 8A). Interestingly, noggin also showed a slight induction; however, only when cells were cultured in control medium but not in the presence of BMPs (Fig. 8B). Expression levels of follistatin were not affected by the gene modulation (data not shown).

DISCUSSION

Adult neural stem cell differentiation is regulated by the interplay of multiple extrinsic and intrinsic factors. Here, we demonstrate that NSCs in both neurogenic niches as well as in neurospheres and isolated NSCs in culture express p57kip2, a protein that we have previously identified as potent negative regulator of myelinating glial cell differentiation (Heinen et al., 2008; Kremer et al., 2009). Our functional data on cultured and transplanted NSCs (derived from both niches) provide strong evidence that p57kip2 additionally acts as an astrogenic determinant and that suppression of this gene strongly interferes with astrocyte generation and promotes the accumulation of OPC characteristics. The observation that glial cell derivatives were not preferentially surviving or

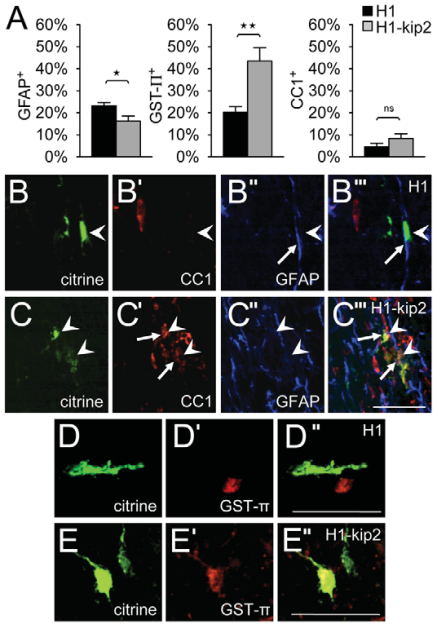

Fig. 7. Transplantation of p57kip2-suppressed NSCs into the intact dorsal rat spinal cord. (**A**) The percentage of GFAP-positive transfected SGZ cells 4 days post-transplantation (only citrine-positive cells were evaluated) was significantly decreased upon p57kip2 suppression. This was accompanied by significantly increased numbers of GST-π-positive cells, as well as increased numbers of CC1-expressing cells. (**B-E‴**) Representative GFAP, GST-π and CC1 immunostaining of control transfected (H1) and p57kip2-suppressed (H1-kip2) SGZ cells upon injection into the white matter. Arrows indicate expressing cells; arrowheads mark absent expression. Data are mean±s.e.m. derived from six operated rats each. *t*-test (**P*<0.01; ***P*<0.01; ns, not significant). Scale bars: 100 μm in C‴; 50 μm in D″,E″.

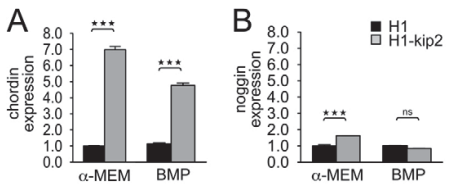

Fig. 8. Gene expression of SGZ-derived adult neural stem cells. NSCs transfected with control (H1) or p57kip2 suppression vectors (H1-kip2) were sorted (FACS). After 7 days in control (α-MEM) or astrocyte-promoting conditions (BMP), cells were lyzed and gene expression levels were determined using quantitative RT-PCR. (**A**,**B**) Chordin transcript levels were strongly increased under both experimental conditions, whereas noggin expression was found to be only slightly upregulated in absence of BMPs. Data are shown as mean±s.e.m. derived from *n*=3 independent experiments. *t*-test (***P<0.001; ns, not significant).

proliferating at higher rates in response to p57kip2 knockdown indicates that p57kip2 is an intrinsic adult neural stem cell regulator and that it plays a role in glial fate decision. During development, p57kip2 was shown to inhibit Mash1 (Ascl1) expression and to prevent neuronal differentiation of embryonal neural stem and progenitor cells (Joseph et al., 2009). No overlap of p57kip2 and neuronal markers such as NeuN was detected in our SGZ sections. Interestingly, in the adult SGZ, Mash1 overexpression led to increased generation of oligodendroglial at the expense of neuronal cells (Jessberger et al., 2008). However, we observed that both SGZ and SVZ cells do not show significantly increased *Mash1* gene expression levels upon p57kip2 knockdown. Furthermore, we found that the number of Mash1-positive cells under our experimental conditions is generally low and not significantly changed in response to altered p57kip2 levels (2.25±1.05% in control cells to 1.9±0.8% in p57kip2-suppressed cells). We therefore conclude that Mash1 regulation is not part of the p57kip2-dependent mechanism and suggest that high levels of Mash1 might artificially impose an oligodendrocyte identity onto precursor cells following overexpression.

As adult CNS NSCs (this study) and OPCs (Kremer et al., 2009) were found to express p57kip2, it will be important to investigate and compare the underlying differentiation mechanisms. This will reveal to what extent similar interactions and binding partners are involved in these separate processes or whether they involve different signaling pathways.

Nature of generated cells

The observation that p57kip2 suppression leads to GFAP reduction might also reflect a loss of stem cell character given its expression among adult neural stem cells in vivo (Doetsch et al., 1999; Laywell et al., 2000). However, dispersed NSCs in culture develop GFAP expression only over time and we demonstrated that both markers GFAP as well as AQP4 are reduced in p57kip2 knockdown cells. AQP4 is a water channel protein located in astroglial end-feet and thus constitutes a specific functional component (Wolburg, 1995). In addition, expression of nestin as a stem cell marker was unaffected upon p57kip2 suppression in NSCs. Therefore, our observations currently support a downregulation of astrocyte rather than of stem cell characteristics.

It remains to be shown whether the modulation of p57kip2 expression exerts a direct effect on oligodendrogenesis, or whether the prevention of astrocyte generation is indirectly promoting it. However, as we did not observe induction of neuronal markers, this would argue for a direct regulation. The appearance of OPC features, but not of late oligodendrocyte markers, as we have observed it, might be a consequence of media composition (serum content or presence of BMPs) and needs to be addressed in future investigations. Nevertheless, such a limited differentiation switch could also be an advantage in the naturally occurring remyelination process in the context of (exogenous) cell replacement. Indeed, transplanted p57kip2 suppressed stem cells were able to induce expression of the OPC marker GST-π and to initiate the expression of the mature marker CC1, indicating that environmental factors can further promote their differentiation. In this regard, long-term observations of transplanted NSCs will show whether these cells are able to distribute in the tissue, successfully interact with axons and restore myelin. Future in vivo studies conducted in a myelin-deficient background such as in the shiverer mouse are likely to provide an answer to this question. As OPC supporting signals might also derive from injured, demyelinated or inflamed tissues, modulated NSCs should then also be transplanted into injured or diseased animals. The observation of a p57kip2 suppression-dependent positive differentiation effect in diseased animals could be of particular importance as the plasticity of the stem cell compartment, especially upon CNS inflammation, was shown to be negatively affected (Pluchino et al., 2008; Wang et al., 2008). In the inflamed demyelinated CNS, as found in rodent experimental autoimmune encephalomyelitis (EAE) or in MS, increased numbers of SVZ-derived progenitors were shown to be mobilized towards lesions and to give rise to new oligodendrocyte precursors (Picard-Riera et al., 2002; Menn et al., 2006; Nait-Oumesmar et al., 2007). This behavior differs from their default neuronal differentiation and indicates that stem cell niches sense altered signals. It will therefore be of importance to analyze whether this shift is reflected by lowered p57kip2 levels in NSCs and whether this could represent an endogenous mechanism to restore myelination.

Underlying molecular mechanisms

Although the underlying mode of action remains to be elucidated in more detail, p57kip2 is likely to interfere with BMP-related signaling cascades. It has been proposed that BMP2 and BMP4 promote astroglial lineage commitment (Gross et al., 1996), and we could demonstrate that suppression of p57kip2 blocks the BMP-dependent astrogenic effect on adult NSCs. It is therefore conceivable that either BMP-receptor expression or activation is affected, or that signaling components further downstream are modulated. Chordin and noggin are potent antagonists of BMP signaling (Bachiller et al., 2000) and we investigated whether p57kip2 is involved in the regulation of their expression. The observed strong induction of chordin and the moderate induction of noggin (Fig. 8) indicate that, upon p57kip2 suppression, these antagonists are produced and that they mediate the anti-astrocyte effects. It is possible that, in the p57kip2-dependent glial fate regulation process, chordin and noggin act as cell-intrinsic safeguards in a BMP-enriched environment, as it was shown to occur upon transplantation into the spinal cord (Setoguchi et al., 2004; Hampton et al., 2007; Matsuura et al., 2008). In addition, the recent demonstration that chordin promotes oligodendrogenesis from adult NSCs in vitro and in vivo (Jablonska et al., 2010) suggests that a cell-autonomous induction of chordin actively contributes to the establishment of oligodendroglial features. However, because in this study chordin infusion induced Mash1 positivity, which we did not observe upon p57kip2 suppression, it remains to be shown whether similar molecular processes are

involved or whether the p57kip2-dependent mechanism exerts a unique mode of action. Whether these molecules account for all observed changes or whether further molecular regulators, such as Id4, Smad4, Smad1/5/8 or Stat, also depend on p57kip2 expression and control the fate of transfected NSCs remains to be shown in future experiments.

Biomedical relevance

Gliosis, i.e. enhanced astrocyte generation, as well as oligodendroglial turnover and remyelination are important features in pathological CNS conditions such as spinal cord injury (SCI) or MS. In both situations, appropriate cell replacement is required in order to restore axonal functions but endogenous regeneration is usually insufficient or skewed towards production of astrocytes. After demyelinating injury, BMPs were shown to promote gliosis (Setoguchi et al., 2001) and subsequent glial scar formation, which interferes with axonal restoration as well as remyelination. Similarly, glial scar formation following spinal cord injury was also shown to be responsible for failure of axonal regeneration (Silver and Miller, 2004). However, supply of appropriate myelinating glial cells such as olfactory ensheathing cells or Schwann cells was reported to enhance the repair process (Li and Raisman, 1995; Barnett et al., 2000). This demonstrates the necessity of reducing the number of astrocytes and simultaneously providing myelinating glial cells in order to either restore axonal function (MS) or to allow axons to grow through the lesion zone (SCI). The observation that p57kip2-suppressed NSCs are directed towards the oligodendroglial lineage and at the same time display significantly reduced cell death rates, indicates that such a modulated cell population could represent a valuable tool for functional cell replacement.

In summary, we provide evidence that the inhibitory regulator of myelinating glial precursor cell differentiation, p57kip2, exerts a strong influence over adult neural stem cell fate decision and might represent an important element that is responsible for glial cell specificity. These findings are not only of interest in terms of endogenous cell turnover and stem cell biology, but they could also provide the basis for future CNS repair strategies that aim at the generation of appropriate cell types, prevention of gliosis and restoration of brain function.

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Author contributions

J.J.J. and F.J.R. performed experiments, data collection and analysis, data interpretation and manuscript writing; A.T. and B.S. performed experiments, data collection and analysis; Mahesh Kandasamy collected data; N.W. and O.A. carried out data collection, analysis and interpretation; H.-P.H. was responsible for conception and design, data interpretation and manuscript writing; L.A. was responsible for conception and design, data analysis and interpretation; P.K. was responsible for conception and design, data analysis and interpretation, manuscript writing, and final approval of the manuscript.

Competing interests statement

The authors declare no competing financial interests.

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2.3 The remyelination Philosopher's Stone: stem and progenitor cell therapies for multiple sclerosis

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Abstract

Multiple sclerosis (MS) is an autoimmune disease that leads to oligodendrocyte loss and subsequent demyelination of the adult central nervous system (CNS). The pathology is characterized by transient phases of recovery during which remyelination can occur as a result of resident oligodendroglial precursor and stem/progenitor cell activation. However, myelin repair efficiency remains low urging the development of new therapeutical approaches that promote remyelination activities. Current MS treatments target primarily the immune system in order to reduce the relapse rate and the formation of inflammatory lesions, whereas no therapies exist in order to regenerate damaged myelin sheaths. During the last few years, several transplantation studies have been conducted with adult neural stem/progenitor cells and glial precursor cells to evaluate their potential to generate mature oligodendrocytes that can remyelinate axons. In parallel, modulation of the endogenous progenitor niche by neural and mesenchymal stem cell transplantation with the aim of promoting CNS progenitor differentiation and myelination has been studied. Here, we summarize these findings and discuss the properties and consequences of the various molecular and cell-mediated remyelination approaches. Moreover, we address age-associated intrinsic cellular changes that might influence the regenerative outcome. We also evaluate the extent to which these experimental treatments might increase the regeneration capacity of the demyelinated human CNS and hence be turned into future therapies.

† These senior authors contributed equally to this work.

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The manuscript writing including all figures was prepared by Janusz Joachim Jadasz and subsequently reviewed, amended and approved by all co-authors.

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2.4 Mesenchymal Stem Cell Conditioning Promotes Rat Oligodendroglial Cell Maturation

Janusz Joachim Jadasz, David Kremer, Peter Göttle, Nevena Tzekova, Julia Domke, Francisco J. Rivera, James Adjaye, Hans-Peter Hartung, Ludwig Aigner, and Patrick Küry

Abstract

Oligodendroglial progenitor/precursor cells (OPCs) represent the main cellular source for the generation of new myelinating oligodendrocytes in the adult central nervous system (CNS). In demyelinating diseases such as multiple sclerosis (MS) myelin repair activities based on recruitment, activation and differentiation of resident OPCs can be observed. However, the overall degree of successful remyelination is limited and the existence of an MS-derived anti-oligodendrogenic milieu prevents OPCs from contributing to myelin repair. It is therefore of considerable interest to understand oligodendroglial homeostasis and maturation processes in order to enable the development of remyelination therapies. Mesenchymal stem cells (MSC) have been shown to exert positive immunomodulatory effects, reduce demyelination, increase neuroprotection and to promote adult neural stem cell differentiation towards the oligodendroglial lineage. We here addressed whether MSC secreted factors can boost the OPC's oligodendrogenic capacity in a myelin non-permissive environment. To this end, we analyzed cellular morphologies, expression and regulation of key factors involved in oligodendroglial fate and maturation of primary rat cells upon incubation with MSC-conditioned medium. This demonstrated that MSC-derived soluble factors promote and accelerate oligodendroglial differentiation, even under astrocytic endorsing conditions. Accelerated maturation resulted in elevated levels of myelin expression, reduced glial fibrillary acidic protein expression and was accompanied by downregulation of prominent inhibitory differentiation factors such as Id2 and Id4. We thus conclude that apart from their suggested application as potential anti-inflammatory and immunomodulatory MS treatment, these cells might also be exploited to support endogenous myelin repair activities.

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Cell preparation, culture and transfection were performed by Janusz Joachim Jadasz and in assistance of listed authors. Janusz Joachim Jadasz designed all experiments. The manuscript writing including all figures was prepared by Janusz Joachim Jadasz together with PK and subsequently reviewed, amended and approved by all co-authors.

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Mesenchymal Stem Cell Conditioning Promotes Rat Oligodendroglial Cell Maturation

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Abstract

Oligodendroglial progenitor/precursor cells (OPCs) represent the main cellular source for the generation of new myelinating oligodendrocytes in the adult central nervous system (CNS). In demyelinating diseases such as multiple sclerosis (MS) myelin repair activities based on recruitment, activation and differentiation of resident OPCs can be observed. However, the overall degree of successful remyelination is limited and the existence of an MS-derived anti-oligodendrogenic milieu prevents OPCs from contributing to myelin repair. It is therefore of considerable interest to understand oligodendroglial homeostasis and maturation processes in order to enable the development of remyelination therapies. Mesenchymal stem cells (MSC) have been shown to exert positive immunomodulatory effects, reduce demyelination, increase neuroprotection and to promote adult neural stem cell differentiation towards the oligodendroglial lineage. We here addressed whether MSC secreted factors can boost the OPC's oligodendrogenic capacity in a myelin non-permissive environment. To this end, we analyzed cellular morphologies, expression and regulation of key factors involved in oligodendroglial fate and maturation of primary rat cells upon incubation with MSC-conditioned medium. This demonstrated that MSC-derived soluble factors promote and accelerate oligodendroglial differentiation, even under astrocytic endorsing conditions. Accelerated maturation resulted in elevated levels of myelin expression, reduced glial fibrillary acidic protein expression and was accompanied by downregulation of prominent inhibitory differentiation factors such as Id2 and Id4. We thus conclude that apart from their suggested application as potential anti-inflammatory and immunomodulatory MS treatment, these cells might also be exploited to support endogenous myelin repair activities.

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Introduction

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS) and a leading cause of fixed neurological disability of young adults in Western countries. MS is mediated by an immune response against myelin sheaths and myelin-producing cells, the oligodendrocytes. Degeneration and loss of myelin leave axons unprotected and slow down or even block saltatory conduction of electrical signals which contributes to clinical impairment. Moreover, naked axons are highly susceptible to the overall inflammatory environment ultimately resulting in neuronal damage and neurodegeneration, the extent of which dictates the level of permanent neurological disability. Although repair activities are generally limited within the adult CNS, a certain degree of remyelination can be observed. This regenerative process is dependent on successful cell replacement, which is mainly mediated via endogenous oligodendroglial progenitor/precursor cell (OPC) activation [1]. Unfortunately, overall remyelination efficiency remains poor due to limited cellular differentiation and migration [2,3] or as a consequence of astrocytic cues, such as bone morphogenetic proteins (BMPs), as they have been shown to instruct progenitor cells to differentiate into glial cells expressing astrocyte characteristics [4].

In light of the increasing number of potent immunomodulatory therapies for MS which allow for efficient control of inflammatory relapse activity and preventing further tissue damage [5], the current focus in exploring new MS treatments has shifted towards neuroprotection and functional tissue repair [6]. Regarding the restoration of axonal connectivity and fast signal transmission, recent experimental studies have unravelled a series of distinct molecular switches responsible for the homeostasis and differentiation of stem- and precursor cells within the inflamed tissue [3]. Despite this increasing knowledge on myelinating cell turnover, an integrative translational therapeutic approach aiming at cell-based glial protection and restoration of myelin integrity remains an unmet therapeutic goal. Thus, apart from limiting or re-dressing the deviated immune response, novel methods to support functional regeneration are required.

Mesenchymal stem cells (MSCs) have been shown to exert positive immunomodulatory effects [7], to reduce demyelination [8], to enhance neuroprotection [9] and to promote adult neural stem cell (aNSC) differentiation towards the oligodendroglial lineage at the expense of astrocytes and neurons [10,11]. Secreted factors remain to be elucidated, with the exception of hepatocyte growth factor (HGF), which has been described as a mediator of recovery in MS models [12]. Moreover, ciliary neurotrophic factor (CNTF) was found to foster stem cell derived oligodendrogenesis but appears not to be an MSC-derived key regulator [13].

Here we investigated to what extent OPCs, as the main cellular source for remyelination, are actively determined and supported in their differentiation process towards myelin building mature oligodendrocytes upon stimulation with soluble mesenchymal factors. We found that MSC-conditioned medium supports oligodendroglial fate decision under astrocytic endorsing conditions enhancing the expression of differentiation markers and promoting morphological maturation. Accelerated maturation featured elevated levels of 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and myelin basic protein (MBP) expression and reduced glial fibrillary acidic protein (GFAP) expression. This process was accompanied by downregulation of prominent inhibitory differentiation factors such as Id2 and Id4 and could not be mimicked by HGF. We thus conclude that apart from their suggested role as a prospective anti-inflammatory MS treatment these cells might also be applied in order to support endogenous myelin repair activities.

Materials and Methods

Ethics Statement

All cell preparations and all animal care were carried out in accordance with the European Communities Council Directive (86/609/EEC). Furthermore, we received from the ethics committee of the animal research facility of the Heinrich-Heine-University permits to kill animals and to preserve tissues (O69/2011; O118/11). Ethical considerations and details on the generation of primary oligodendroglial cells as well as of mesenchymal stem cells have previously been reported by us [10,14–16].

Mesenchymal Stem Cell Preparation and Medium Conditioning

Bone marrow plugs were harvested from femural and tibial bone of 2–4 month-old female Fisher rats (Charles River Deutschland GmbH, Germany). Adult rats were anesthetized using ISOFLURAN (DeltaSelect, Langenfeld, Germany) and killed by decapitation. Plugs were mechanically dissociated in Minimum Essential Medium alpha Medium (α-MEM) (Gibco Cell Culture, Life Technologies, Germany) and recovered by centrifugation. Cell pellets were resuspended in α-MEM containing 10% fetal bovine serum (α-MEM-10%FBS; PAN Biotech GmbH, Germany) and seeded at 1×10^6 cells/cm^2 in a humidified incubator at 37°C with 5% CO_2. After three days, the entire culture was used and designated as bone marrow cells (BMCs) or separated into non-adherent cells and adherent cells. Adherent cells were incubated in fresh **α**-MEM-10%FBS as standard/control medium (designated as α-MEM from here on) until a confluent layer of cells was reached. These cells were trypsinized using a 0.25% Trypsin-EDTA solution (Gibco Cell Culture, Life Technologies, Germany), seeded in α-MEM-10%FBS at 8,000 cells/cm^2. After 3–5 days of culture, the resulting monolayer of cells, hereafter named rat bone marrow-derived mesenchymal stem cells (MSCs), was trypsinized and frozen or further cultured

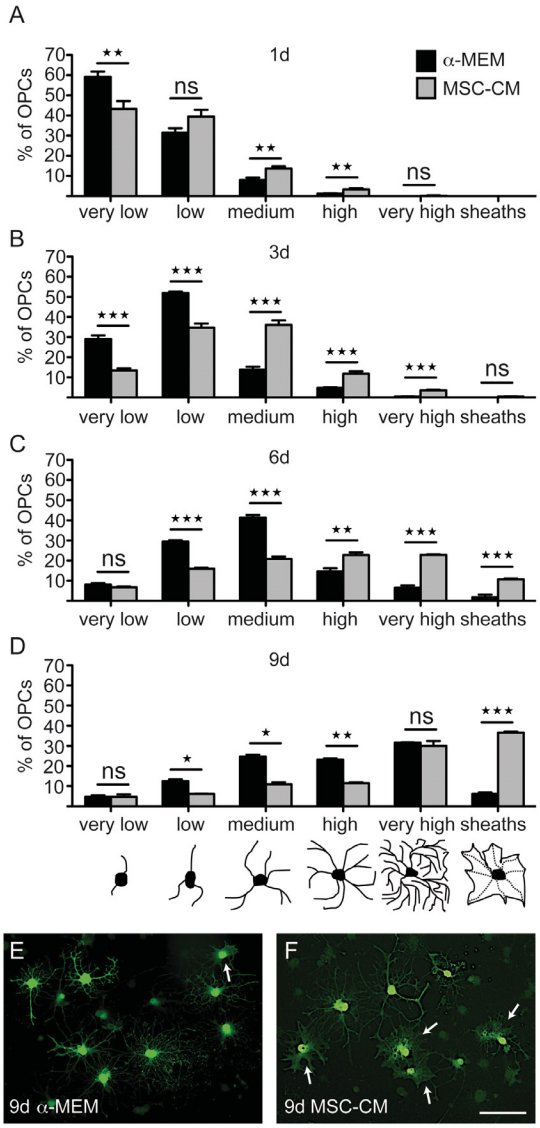

Figure 1. MSC-CM stimulates morphological maturation of OPCs. Mesenchymal stem cell conditioned medium (MSC-CM) accelerates maturation of cultured oligodendroglial progenitors cells. We identified six distinct morphologies (see bottom), from a very low number of processes in progenitor cells to multiple process-bearing cells (low, medium, high) to mature cells with a very high degree of arborisation or flattened appearance (sheaths). (**A–D**) Analysis of OPC morphology distribution revealed an MSC-CM-dependent shift towards more mature cells (black bars **α**-MEM treated cells; grey bars MSC-CM stimulated cells) after one (**A**), three (**B**), six (**C**) and nine days (**D**). (**E,F**) Representative citrine labelled OPCs revealing advanced morphologies and promoted sheath formation (arrows) upon MSC-CM stimulation after nine days. Data are shown as mean values ± SEM derived from n = 5 experiments. t-test (ns: not significant, * $P<0.05$; ** $P<0.01$; *** $P<0.001$). Scale bar: 50 µm.
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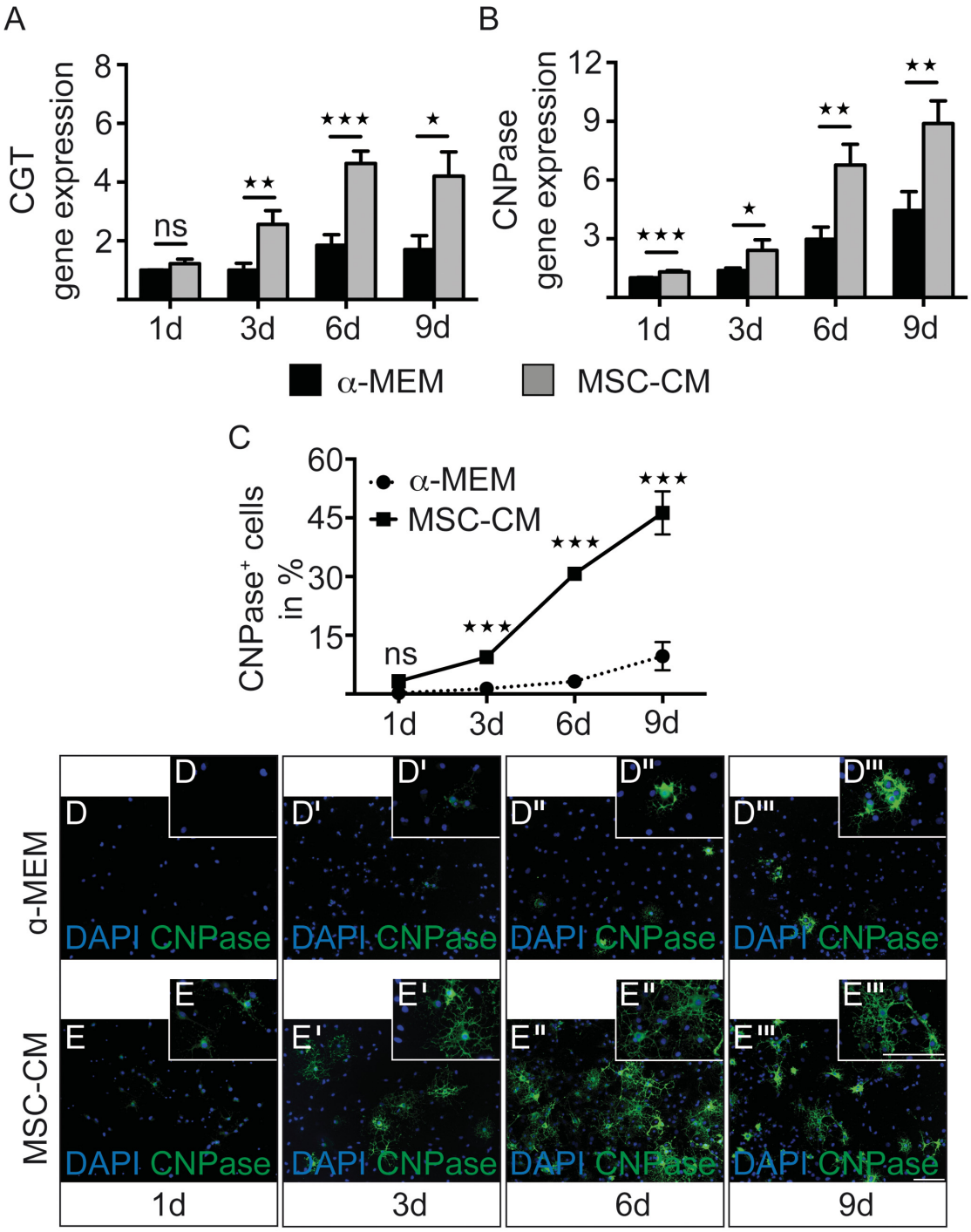

Figure 2. MSC-CM leads to enhanced early myelin expression. Determination of transcript levels by means of quantitative real-time RT-PCR. (**A**) Upregulation of ceramide galactosyltransferase (CGT) expression was detected after three, six and nine days in culture, (**B**) whereas gene expression levels of CNPase were elevated at every measured time point. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as reference. (**C**) In addition, an increased percentage of CNPase-expressing OPCs was observed among MSC-CM treated cells as compared to cells

grown in α-MEM. Significant differences were detected from day three onwards. (**D–E‴**) Representative immunofluorescent stainings of CNPase expressing OPCs at all four-time points of investigation. Data are shown as mean values ± SEM and derive from n = 8 (CGT), n = 8 (CNPase, q-RT-PCR) and n = 4 (CNPase, immunostainings) experiments. t-test (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). Scale bars: 50 µm.
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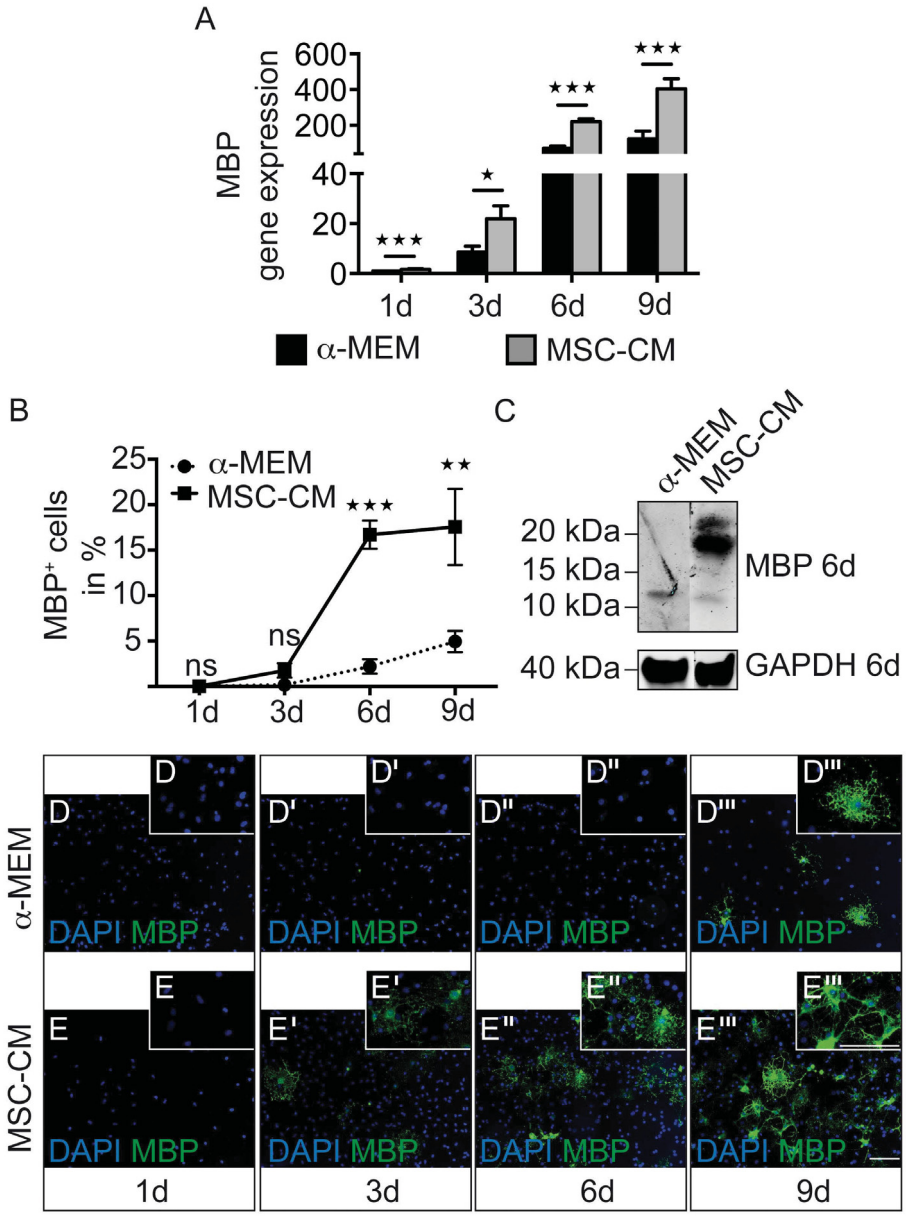

Figure 3. MSC-CM enhances myelin basic protein expression. (**A**) Gene expression levels of MBP are up-regulated after one, three, six and nine days in MSC-CM. (**B**) Determination of the degree of MBP-positive OPCs as revealed by immunostainings show a significantly increased number after MSC-CM treatment for six and nine days. (**C**) Western blot analysis shows increased MBP protein levels after six days in mesenchymal stem cell conditioned medium. GAPDH was used as internal reference. (**D–E‴**) Representative immunofluorescent stainings of MBP expressing OPCs at all four time points of investigation. Data are shown as mean values ± SEM derived from n = 6 (q-RT-PCR) and n = 3 (immunostainings, Western Blot) experiments. t-test (* $P<0.05$; ** $P<0.01$, *** $P<0.001$). Scale bars: 50 µm.
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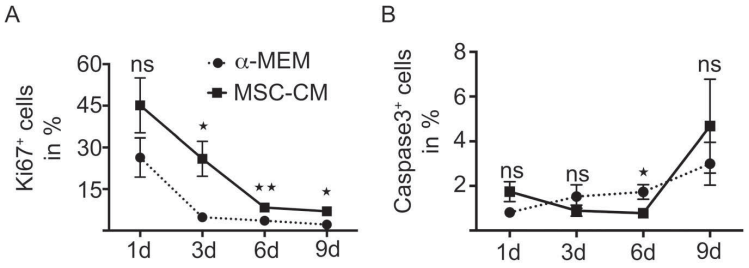

Figure 4. MSC-CM treatment enhances OPC proliferation, while cell death rate remains low. (**A**) Anti-Ki67 immunofluorescent stainings and evaluation of positive cells revealed a higher proliferation rate under MSC-CM treatment as compared to α-MEM, whereas (**B**) cell death rates remained low under both conditions with a minor significant survival effect upon MSC-CM treatment at six days. Data are shown as mean values ± SEM derived from n = 4 experiments. t-test (ns: not significant, * P<0.05; **P<0.01).
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for experiments. MSC-conditioned medium (MSC-CM) was prepared by plating 12,000 MSCs per cm^2 and incubation in α-MEM-10% FBS or α-MEM-1% FBS for three days. Conditioned media were not diluted and directly applied to OPCs 4 h to 6 h after plating. HGF (R&D Systems, Minneapolis, MN) was applied at a final concentration of 50 ng/ml in α-MEM-10% FBS according to Bai and colleagues [12]. For HGF neutralization experiments, α-MEM-10% FBS and MSC-CM were incubated at 37°C with a final concentration of 10 µg/ml function-blocking anti-HGF antibody (R&D Systems) for 1 h before use as previously published [12].

Oligodendroglial Cell Culture

Purification and culturing of OPCs was performed according to earlier descriptions [17]. Briefly, P1 rats were anesthetized using ISOFLURAN and killed by decapitation. Dissociated P1 rat cortices were cultured on poly-D-lysine (PDL)-coated cell culture flasks in DMEM substituted with 10% FBS and 4 mM L-glutamine. After 10 days, flasks were shaken at 250 rev/min for 2 h to deplete from microglial contamination. Then flasks were shaken for another 20 h in which OPCs were dislodged from the underlying astrocyte-layer and replated on PDL-coated culture dishes or glass cover slips in high glucose DMEM-Sato-based medium containing bovine 5 µg/mL insulin, 50 µg/mL human

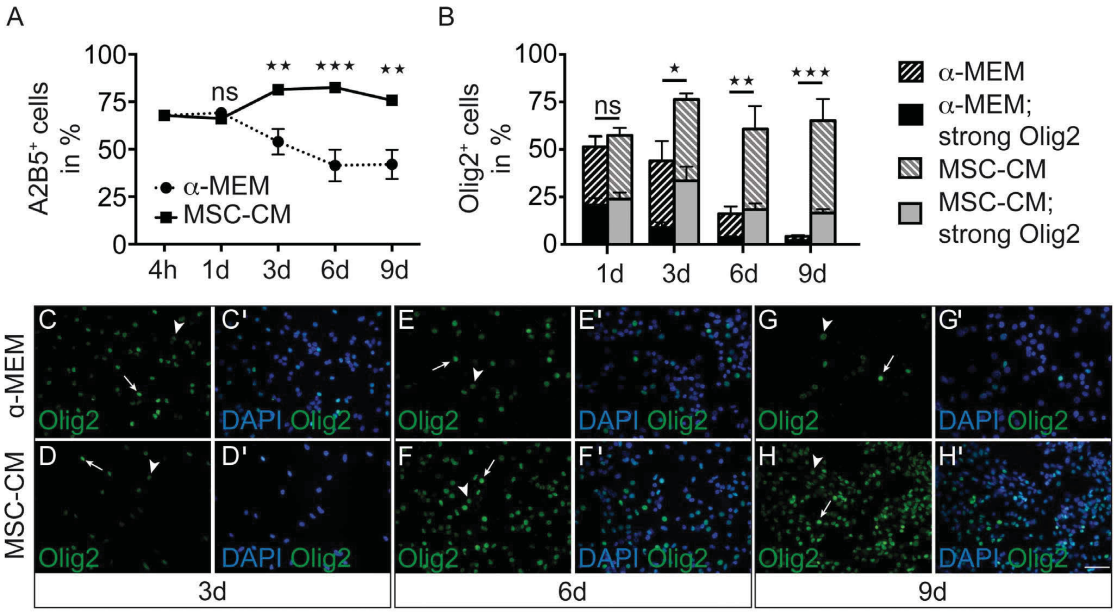

Figure 5. MSC-CM dependent regulation of early OPC markers. (**A**) Determination of the degree of A2B5 positive cells as revealed by immunofluorescence stainings. α-MEM and MSC-CM were given to the cells after four hours. (**B**) Determination of the degree of weak and strong Olig2 expressing cells during the course of α-MEM and MSC-CM stimulation up to nine days. (**C–H'**) Representative immunofluorescent stainings of Olig2 expressing OPCs at all three time points. Arrowheads mark weak expressing cells, whereas arrows point to strong expressors. Data are shown as mean values ± SEM derived from n = 3 (A2B5) and n = 4 (Olig2) experiments. t-test (ns: not significant, * P<0.05; **P<0.01; *** P<0.001). Scale bar: 50 µm.
doi:10.1371/journal.pone.0071814.g005

transferrin, 100 µg/mL BSA, 6.2 µg/mL progesterone, 16 µg/mL putrescine, 5 ng/mL sodium selenite, and 4 mM L-glutamine (all Sigma-Aldrich, Missouri, USA). After cell sedimentation (4 to 6 h after plating) the medium was changed after one washing step with PBS to control medium or MSC-CM (no further dilution), which was changed every third day. For morphological staging, OPCs were transfected with a citrine expression vector and morphologies were determined by means of a morphological key as previously described in [14,15].

RNA Preparation, cDNA Synthesis and Quantitative Reverse Transcription (RT)-PCR

Prepared OPCs were cultured for up to nine days in α-MEM or MSC-CM. Total RNA purification from cells was done using the RNeasy procedure (Qiagen, Hilden, Germany). Isolated RNA was reverse transcribed using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative determination of gene expression levels was performed on a 7900HT sequence detection system (Life Technologies, Applied Biosystems, Darmstadt, Germany) using Power SybrGreen universal master mix (Applied Biosystems). Primer sequences were determined using PrimerExpress 2.0 software (Applied Biosystems) and tested for the generation of specific amplicons: GFAP_fwd: CTG GTG TGG AGT GCC TTC GT, GFAP_rev: CAC CAA CCA GCT TCC GAG AG, CGT_fwd: CCG GCC ACC CTG TCA AT, CGT_rev: CAG GGA GAC GAG TCA CAA CGT, CNPase_fwd: CTG CCG CCG GGA CAT, CNPase_rev: TCC CGC TCG TGG TTG GTA T, MBP_fwd: CAA TGG ACC CGA CAG GAA AC, MBP_rev: TGG CAT CTC CAG CGT GTT C, Id2_fwd: AGA ACC AAA CGT CCA GGA CG, Id2_rev: TGC TGA TGT CCG TGT TCA GG, Id4_fwd: CAG CTG CAG GTC CAG GAT GT,

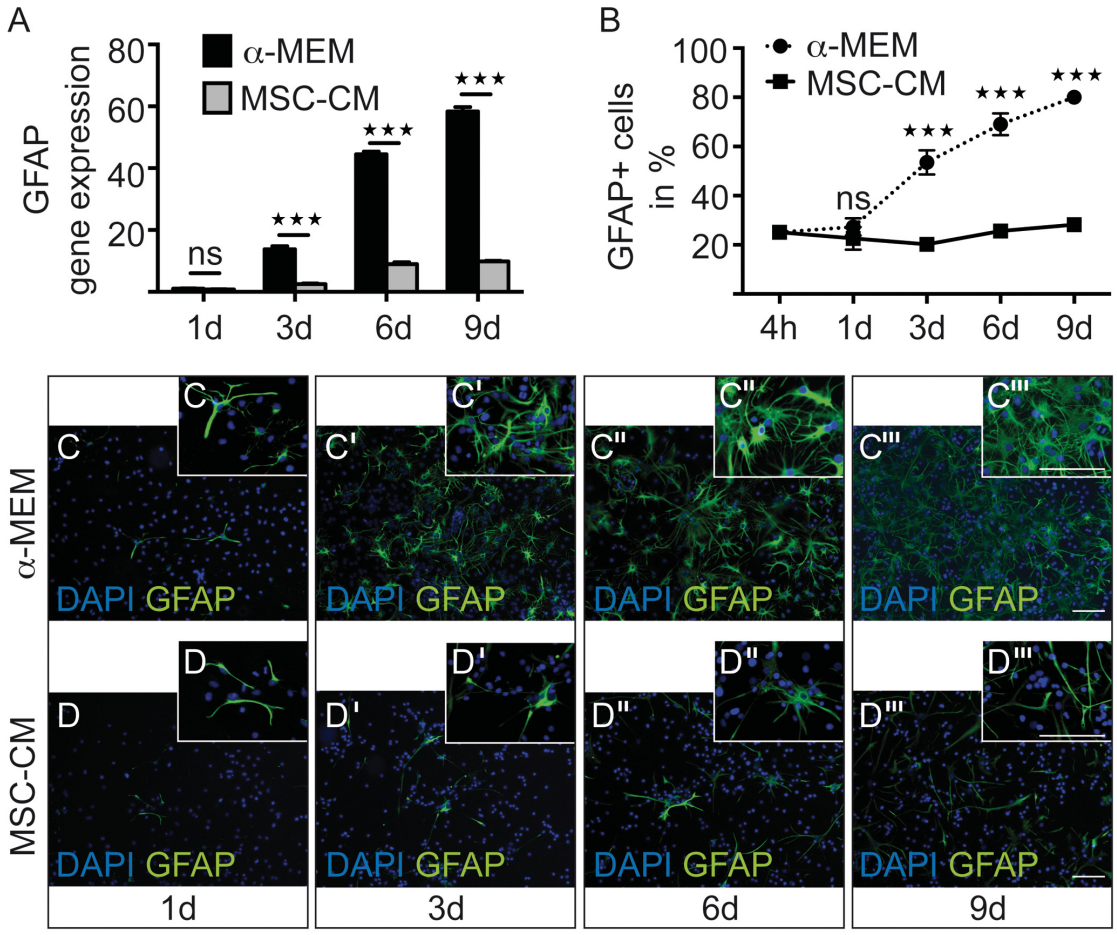

Figure 6. Downregulation of GFAP expression following stimulation with MSC-CM. OPCs were plated for four hours in SATO medium before changing the medium to control (α-MEM) or to mesenchymal stem cell conditioned medium (MSC-CM) and analysis after one, three, six and nine days. (**A**) Consistently decreased GFAP transcript levels were detected at every time point using quantitative real-time RT-PCR. (**B**) Determination of the degree of GFAP-positive OPCs revealed increasing numbers among α-MEM treated cells whereas MSC-CM stimulation stabilized low GFAP expression levels. (**C–D'''**) Representative anti-GFAP immunofluorescent stainings. Data are shown as mean values ± SEM derived from n = 3 for both, q-RT-PCR as well as immunostaining experiments. t-test (*** $P<0.001$). Scale bars: 50 µm.
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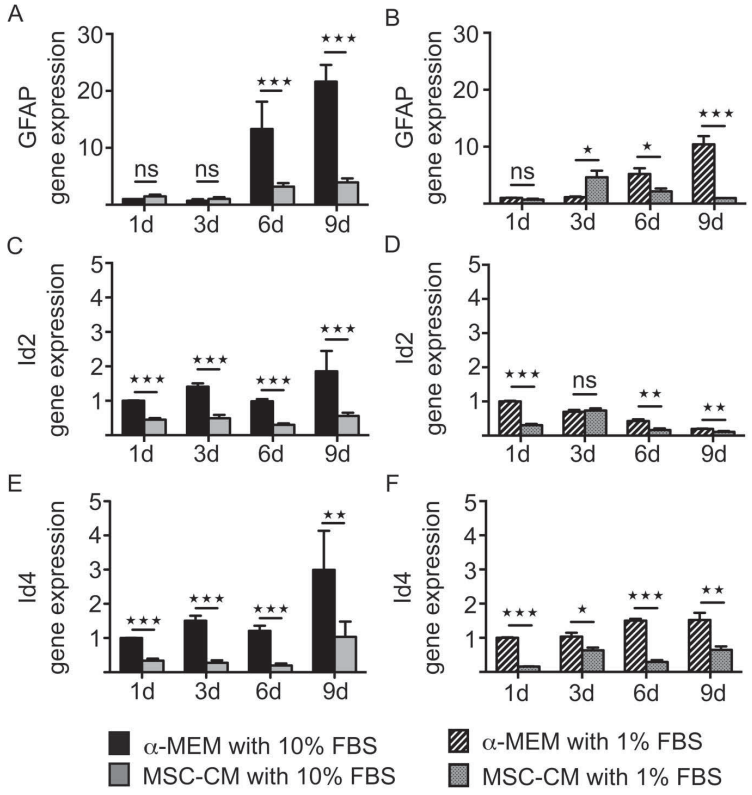

Figure 7. Serum reduced conditioned medium. Determination of transcript levels by means of quantitative real-time RT-PCR. Investigation of GFAP, Id2 and Id4 gene expression levels in α-MEM versus MSC-CM containing 10% FBS (**A,C,E**). Investigation of GFAP, Id2 and Id4 gene expression levels in α-MEM versus MSC-CM containing 1% FBS (**B,D,F**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as reference gene; data are shown as mean values ± SEM derived from n = 7 for Id2, Id4 and n = 3 for GFAP (**A,C,E**) and n = 3 (**B,D,F**) experiments. t-test (ns: not significant, * $P<0.05$; ** $P<0.01$; *** $P<0.001$).
doi:10.1371/journal.pone.0071814.g007

Id4_rev: AAA GTG GAG ATC CTG CAG CAC, GAPDH_fwd: GAA CGG GAA GCT CAC TGG C, GAPDH_rev: GCA TGT CAG ATC CAC AAC GG, ODC_fwd: GGT TCC AGA GGC CAA ACA TC, ODC_rev: GTT GCC ACA TTG ACC GTG AC. GAPDH and ODC were used as reference genes, and relative gene expression levels were determined according to the ΔΔCt method (Applied Biosystems). Each sample was measured in quadruplicate; data are shown as mean values ± SEM and t-test was applied in order to determine statistical significance (Prism 5.0c; GraphPad Software).

Immunohistochemical Procedures

For marker expression analysis, OPCs were fixed with 4% paraformaldehyde/PBS solution, PBS washed, blocked for 45 minutes using 1% normal goat serum and 1% Triton in PBS and subjected to antibody incubation at 4°C overnight: mouse anti-myelin basic protein (MBP) (1:500, Sternberger Monoclonals, Lutherville, MD), mouse anti-2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (1:500, Sternberger Monoclonals), rabbit anti-cleaved Caspase3 (1:500, Cell Signaling Technology, Leiden, The Netherlands), rabbit anti-Ki67 (1:500, Millipore, Schwalbach, Germany), rabbit anti-GFAP (1:1000, Dako, Hamburg, Germany), mouse anti-GFAP (1:1000, Millipore), mouse anti-A2B5 (1:200, Millipore) and rabbit anti-Olig2 (1:1000, Millipore). Following PBS washes secondary anti-mouse and anti-rabbit antibodies conjugated with either Alexa Fluor594 or Alexa Fluor488 (1:1000, Invitrogen) were added for 2 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Roche). Cells were mounted under Citifluor (Citifluor, Leicester, UK) and analyzed using an Axio Cam HRc microscope (Zeiss, Jena, Germany). HGF-ELISA was performed using Mouse/Rat HGF Quantikine ELISA kit according to the protocol of the supplier (R&D Systems). Absolute cell numbers were determined using the nucleus counter macro of the ImageJ 1.46e software.

Western Blot Analysis

Lysis of control and MSC-CM treated OPCs was carried out on ice with radioimmu-noprecipitation assay buffer (RIPA buffer; Cell Signaling Technology, Danvers, Massachusetts, USA) with addition of Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Specimens were subjected to two sonification cycles of 15 s each, and protein solutions were kept on ice for 1 min between the pulses. Protein concentrations were determined using the DC Protein Assay (BioRad, München, Germany). Probes were subjected to standard SDS gel electrophoresis and

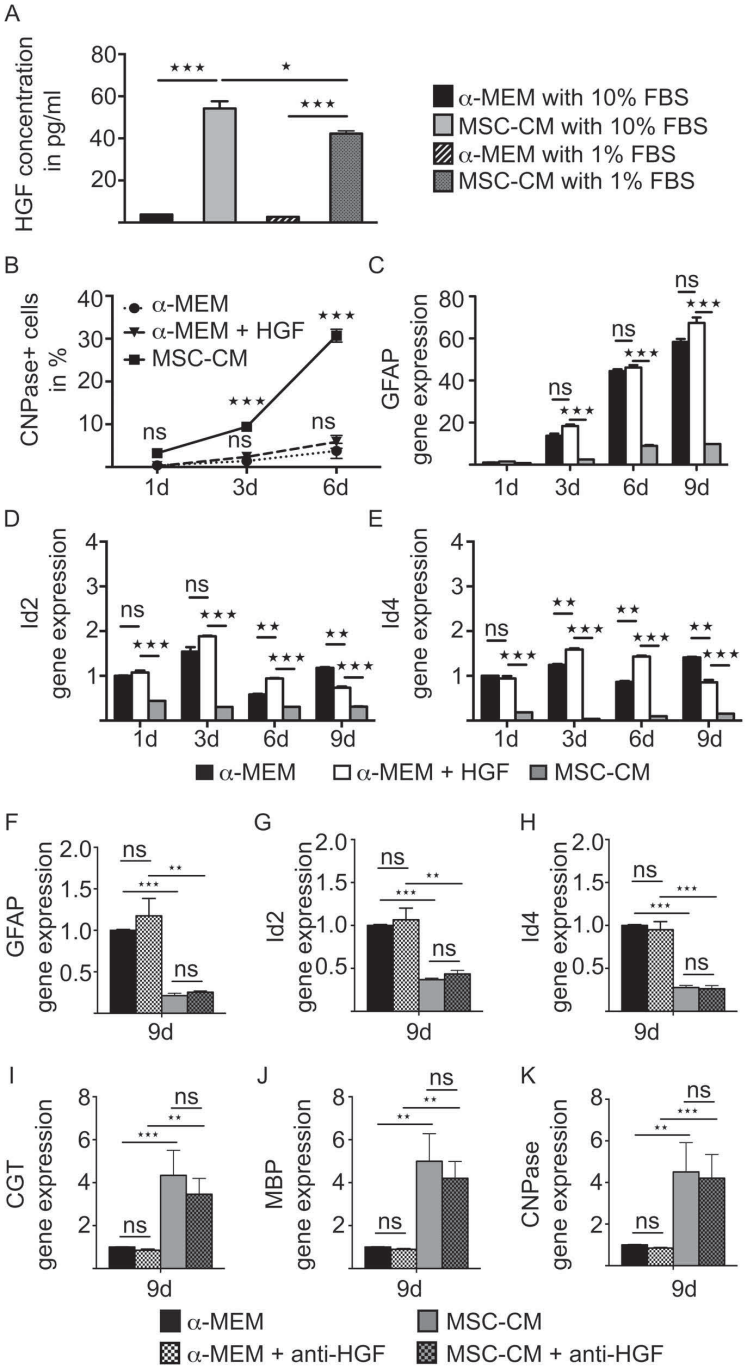

Figure 8. HGF does not mimic MSC-CM mediated cellular effects. (**A**) Determination of HGF protein content in stimulation media by means of ELISA revealing a robust increase upon MSC conditioning. (**B**) No difference regarding CNPase positivity was observed among OPCs grown in **α**-MEM in the absence or presence of recombinant HGF, whereas MSC-CM reproducibly increased CNPase expression. (**C–E**) Determination of transcript levels by means of quantitative real-time RT-PCR. No significant differences regarding Id2, Id4 and GFAP transcript levels were observed among OPCs grown in α-MEM in the absence or presence of recombinant HGF. MSC-CM treatment significantly reduced transcript levels of all three genes at all time points. (**F–K**) Anti-HGF antibody mediated depletion experiments revealed no effect on astrocyte (GFAP, Id2, Id4) and oligodendroglial/myelin

(CGT, MBP, CNPase) gene expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as reference gene. All data are shown as mean values ± SEM derived from n = 3 experiments for each analysis. t-test (ns: not significant, * $P<0.05$; ** $P<0.01$; *** $P<0.001$).
doi:10.1371/journal.pone.0071814.g008

Western blotting using RunBlue SDS gels (Expedeon, Cambridgeshire, UK), RunBlue Blot Sandwich nitrocellulose (Expedeon) applying mouse anti-MBP (MBP; Covance; 1:500, Princeton, New Jersey, USA), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore; 1:1000) antibodies. Signals were visualized using IRDye 680LT donkey anti-mouse and IRDye 800CW donkey anti-mouse antibodies (1:15000) and an Odyssey infrared imaging system scanner (both LI-COR Biosciences, Lincoln, NE). Protein quantifications were performed using the Odyssey software.

Results

Mesenchymal Factors Promote Morphological Maturation of Oligodendroglial Progenitor Cells

We have previously shown that co-culture of bone marrow derived MSCs and aNSCs instructs neural stem cells to become oligodendroglial. This process could be successfully mimicked by the application of mesenchymal stem cell conditioned medium [10]. In order to investigate mesenchymal mediated effects on oligodendroglial cells, OPCs were grown in control condition (non-conditioned α-MEM) or mesenchymal stem cell conditioned medium (MSC-CM) and the distribution of cellular morphologies was determined as described previously [14,15]. We discriminated between early progenitor stages with low numbers of processes (“very low” and “low”), more differentiated cellular stages with increased number of processes and the occurrence of branches (“medium” and “high”), cells with elaborate branching patterns (“very high”) and cells with a flattened appearance featuring sheath formation. Phenotype scoring was performed after one, three, six and nine days of treatment. Absolute numbers of counted cells for all experiments are shown in Table S1. We detected a consistent shift towards the establishment of more complex morphologies at all time points among MSC-CM stimulated cells as compared to control cells (Fig. 1). Of note, at late time points a markedly increased degree of sheath forming cells was detected (Fig. 1D,E,F) indicating that secreted mesenchymal stem cell factors exert a positive effect on oligodendroglial maturation kinetics as well as on process fusion and sheath generation.

MSC-CM Leads to Increased Levels of Myelin Expression

A main component of oligodendroglial cell maturation is the expression of myelin markers. We therefore evaluated transcript levels of the ceramide galactosyltransferase (CGT) gene, which encodes an enzyme known to synthesize the myelin component galactosylceramide (GalC). CGT was significantly increased by 3-fold in OPCs after MSC-CM treatment for three days and by 2.5-fold after six and nine days compared to control (Fig. 2A). Additionally, transcript levels of the early myelin marker CNPase were induced from one to nine days of treatment (Fig. 2B). To evaluate, whether CNPase protein levels were also elevated, we stained and determined the percentage of CNPase positive cells in both conditions. This demonstrated that in OPC populations treated with MSC-CM, CNPase protein expression is strongly and significantly increased at time points three, six and nine days after stimulation (Fig. 2C–E’’’).

Likewise the degree of MBP-expressing OPCs was elevated with significant changes on gene expression levels at every time-point (Fig. 3A), and after six and nine days of treatment for protein levels (Fig. 3B,D–E’’’) as well as in Western blot analysis (Fig. 3C). To confirm that these observed effects were not based on selection processes, we examined proliferation and cell death of control medium and MSC-CM treated OPCs. During the culture of OPCs, proliferation rates gradually decreased but were slightly increased in the presence of mesenchymal secreted factors with significant alterations found after three, six and nine days (Fig. 4A). The percentage of Caspase3-positive OPCs between the two conditions was only altered significantly on day six, though the overall Caspase3-positivity remained low (Fig. 4B).

In order to determine, whether mesenchymal stem cell-derived factors affect oligodendroglial fate decision and stability, we investigated expression of early markers such as A2B5 and Olig2. These experiments confirmed that the majority of plated cells are A2B5 positive and that the expression is decreased under **α**-MEM conditions whereas it is increased upon MSC-CM treatment (Fig. 5A). Likewise, MSC-CM treatment increased the percentage of both weak as well as of strong Olig2 expressors as opposed to strongly decreased levels under **α**-MEM conditions (Fig. 5B–H’).

Regulation of Astroglial Factors

We next analyzed, whether the astrocyte marker glial fibrillary acidic protein (GFAP) was regulated by MSC-CM. Indeed, OPCs incubated with MSC-CM displayed strongly reduced GFAP transcript levels and a significantly lowered degree of GFAP positivity (Fig. 6). This was in contrast to cells grown in **α**-MEM featuring a gradual increase in GFAP protein expression over time, thereby accumulating astrocytic features and gaining astrocytic morphologies. In accordance to Ki67 and Caspase-3 data presented above (Fig. 4), we conclude that the increase of GFAP positive cells in **α**-MEM cannot derive from either increased proliferation or promoted survival of astrocytic cells. Moreover, given the known astrogliogenic potential of high serum containing media, we generated MSC-CM based on **α**-MEM containing 1% FBS only (instead of 10% FBS as shown in Fig. 6). We directly compared gene expression levels in high an low serum containing media and observed that although the astrogliogenic pressure is lower under serum-reduced conditions, MSC-CM can still efficiently counteract the induction of astrocytic features. However, the extent and temporal profiles of GFAP expression revealed to be slightly different (Fig. 7A compare to Fig. 7B).

Initially described as O-2A cells [18], OPCs exert a certain degree of multipotency which under particular circumstances can also give rise to astrocytes [19]. Promotion of astrogliosis has also been reported for the **α**-MEM medium which was applied in this study in particular due to the presence of fetal bovine serum (FBS) [10,20,21]. Furthermore, transcriptional regulators such as inhibitor of differentiation 2 (Id2) and inhibitor of differentiation 4 (Id4) have been described to control glial differentiation acting also as inhibitors of myelin expression [22,23]. We therefore examined whether MSC-CM plays a role in fate decision of immature OPCs via regulation of Id2/4 expression levels and detected significantly decreased Id2 an Id4 transcript levels in OPCs upon MSC-CM stimulation at all time points (Fig. 7C,E). Of note, similar Id regulations were observed when low serum MSC-CM was applied (Fig. 7D,F). However, due to the lowered astrogliogenic potential of serum reduced medium, gene expression levels were lower as compared to the 10% FBS containing media.

Hepatocyte Growth Factor as a Secreted MSC-CM Factor is not the Critical Component for OPC Differentiation

The composition of mesenchymal stem cell secreted factors and their molecular interactions are still poorly understood. Among the few identified molecules is hepatocyte growth factor (HGF), which has been shown to mediate mesenchymal stem cell-induced recovery in animal MS models [12]. We therefore evaluated whether HGF is also a key regulator of the described MSC-CM effect exerted on immature OPCs. To this end, we determined HGF content using enzyme linked immunosorbent assay (ELISA) revealing a significant enrichment of HGF in MSC-CM after a three days incubation of MSCs with **α**-MEM containing either 10% or 1% FBS (Fig. 8A). To clarify, whether HGF exerts an oligodendroglial differentiation effect, we quantified CNPase-positive cells in **α**-MEM supplemented with 50 ng/ml recombinant HGF. Importantly, we did not observe any significant differences between **α**-MEM cultured with or without HGF whereas both conditions induced significantly lower CNPase expression levels compared to MSC-CM (Fig. 8B). Moreover, GFAP expression levels of cells grown in **α**-MEM plus HGF were similar to those kept in **α**-MEM (Fig. 8C). Likewise, Id2/4 transcript levels were also not down-regulated by HFG, as compared to **α**-MEM grown cells, and were even further increased after three and six days in culture (Fig. 8D,E). We also analyzed gene expression levels after neutralizing HGF with a function-blocking antibody based on previously published conditions [12]. We analyzed GFAP, Id2, Id4, CGT, MBP and CNPase gene expression levels at time-point nine days of stimulation. Importantly, we did not detect any differences between media without and media with anti-HGF antibodies (Fig. 8F–K), further supporting that HGF is not involved in the MSC-CM dependent fate and differentiation effect.

Discussion

Oligodendroglial cell maturation is coordinated by different factors either stimulating or inhibiting differentiation processes. Here, we demonstrate that mesenchymal stem cell secreted factors of yet unknown identity support and enforce cell fate decisions and promote differentiation and maturation towards oligodendrocytes. Furthermore, a slight MSC-CM mediated increase in OPC proliferation was observed which is in line with previous data on the control of neural stem cell proliferation [24].

The observation that OPCs did not develop astrocytic features and morphologies in the presence of mesenchymal stem cell-secreted components leads to the assumption that cell fate decisions are regulated. Although currently unidentified, this mixture of factors apparently influences glial transcription patterns – some of the regulated genes being dependent on Olig2 and Id-modulated bHLH transcriptional activities. In this regard, the regulation of Id2 and Id4 is most noteworthy as, for the first time, it demonstrates a glial cell regulatory effect based on mesenchymal stem cells independent of FBS concentration in the medium used for conditioning. Since adding as well as neutralizing HGF turned out to be ineffective in either reversing the fate of fetal bovine serum containing medium instructed cells or in supporting oligodendroglial differentiation, future investigations will have to address the nature of the underlying conditioning factors. It is likely that it will be a cocktail of different ingredients that proves to be essential with perhaps different combinations acting on fate decisions while others influence particular maturation steps. Moreover, the finding that HGF is not the responsible trophic substance is in agreement with previous findings on an HGF-dependent blockade under oligodendroglial differentiation permissive conditions [25]. Of note, we have previously described that another MSC-derived factor, ciliary neurotrophic factor (CNTF), is also not part of the soluble activity acting on aNSCs [13].

The finding of an MSC-CM-dependent counteracting of astrocytic cues leads to the hypothesis that mesenchymal stem cells have the potential to also activate resident stem- and precursor cells upon therapeutic transplantation (locally or systemically). In light of the fact that in contrast to niche-derived NSCs, resident OPCs represent a widespread and dispersed cell population in the CNS promotion of endogenous repair activities by activated and fate-stabilized OPCs provides important advantages. Together with the proliferative enhancement of early progenitor cells and in addition to their proven immunomodulatory effects, these features could indeed contribute to successful myelin repair *in vivo*. Of note, bone morphogenetic protein (BMP) signalling leading to increased Id4 and decreased Olig2 expression has been shown to cause astrocyte differentiation of OPCs [26]. Additionally, after demyelinating injuries, BMPs were shown to promote gliosis [27] and subsequent glial scar formation which interferes with axonal restoration as well as remyelination. Our findings therefore also suggest that MSC-CM pre-treated exogenous OPCs could be a valuable tool for functional exogenous cell replacement helping to withstand astrocytic lesion cues.

Lindsay and colleagues recently demonstrated that conditioning effects may differ between MSCs derived from different tissues, with human mesenchymal stem cells from the lamina propria having the ability to enhance *in vitro* myelination whereas bone marrow-derived cells failed to do so [28]. However, their study focused on oligodendroglial cells which had been cultured for seven days in medium supplemented with growth factors and which are therefore most likely more advanced in terms of fate stabilisation and maturation as well as on olfactory ensheathing cells (OECs). Nevertheless, this study suggests that mesenchymal cocktails are origin-specific, a notion that might be experimentally explored in the future for the identification of active components.

In conclusion, our study provides strong evidence for cell fate decision- as well as differentiation promoting properties of secreted mesenchymal stem cell factors acting on OPCs. As these resident progenitor cells constitute the major source for myelinating glial cell replacement in demyelinating CNS diseases, stimulation of endogenous repair activity by means of MSC transplantation or administration of responsible underlying trophic factors represents an attractive therapeutic approach.

Supporting Information

Table S1 Absolute numbers of counted cells in this study. For each counting at least three different experiments per condition were analyzed.
(XLSX)

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Author Contributions

Conceived and designed the experiments: JJJ DK PG NT FJR HPH LA PK. Performed the experiments: JJJ DK PG NT JD FJR. Analyzed the data: JJJ DK PG NT JD FJR HPH LA PK. Contributed reagents/materials/analysis tools: JJJ FJR JA LA PK. Wrote the paper: JJJ JA HPH LA PK.

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Chapter 3

Discussion

It was long believed that the adult CNS was unable to regenerate and lacks self-renewing cells. However, the discovery of a pool of undifferentiated dividing cells within the brain represents a breakthrough in this conviction. Nowadays it is widely accepted that new cells are born in distinct stem cell niches such as the subgranular zone of the dentate gyrus in the hippocampus and the lateral ventricle of the subventricular zone. Interestingly, the capacity of the brain to generate cells in these niches remains intact throughout life and is responsible for cell turnover even in individuals of higher ages. For instance, every day 700 new neurons are born in the human hippocampus [Spalding et al., 2013]. The question of how self-renewing stem cells can turn into neurons or other cell types, such as astrocytes or myelin-forming oligodendrocytes, and how these newborn cells are integrated in existing structures is largely unsolved. Nevertheless, it is known that brain homeostasis, i.e. maintenance of cellular function of brain cells and regions, depends on the plasticity of existing neuronal cells as well as on the generation of new neurons, astrocytes and oligodendrocytes. Under pathological conditions, these mechanisms seem to be either impaired or insufficient to restore normal brain function. For example, in multiple sclerosis both auto-reactive destruction of myelin and oligodendrocyte cell death occur. After oligodendrocytes and myelin have been destroyed, a phase of remyelination starts. Here, resident OPCs and aNSCs direct their fate into myelin-producing oligodendrocytes and replace destroyed cells. However, this self-repair process is inefficient because, for reasons that are still unclear, oligodendroglial cells lose their myelin producing potential in the pathological milieu of MS lesions. A further problem is that not all resident stem cells turn into oligodendrocytes and that lesioned areas display strong local astrogenic

cues inhibiting oligodendroglial development. Furthermore, this inefficient natural remyelination declines with age and disease progression. Consequently, development of new therapeutic strategies aiming at cell replacement is urgently required. Therefore, to restore proper brain function in MS we need to address the question of whether it is possible to i) increase the number of oligodendrocytes by modulating the cell fate decision of endogenous aNSCs and OPCs, ii) enhance oligodendroglial differentiation and stabilize remyelination by elucidating factors that ameliorate these processes and iii) prevent astroglial differentiation of endogenous or transplanted aNSCs and OPCs.

As shown in this thesis, the intrinsic modulation of $p57^{Kip2}$ facilitates oligodendroglial differentiation of OPCs [Kremer et al., 2009] and stabilizes the fate of aNSCs towards the oligodendroglial lineage, thus increasing absolute numbers of oligodendroglial cells [Jadasz et al., 2012b]. Concomitantly, this intrinsic modulation prevents astroglial differentiation of *in vitro* cultured aNSCs as well as of stem cells transplanted into the adult CNS [Jadasz et al., 2012b]. In addition, extrinsic stimulation of OPCs with mesenchymal stem cell secreted factors was also shown to impede astroglial differentiation [Jadasz et al., 2013]. While it remains unclear which specific factor(s) account for this effect, we were able to further demonstrate that this secreted cocktail consolidates oligodendroglial fate and, at the same time, fosters oligodendrogenic differentiation of OPCs [Jadasz et al., 2013].

3.1 Increasing the number of oligodendrocytes on intrinsic glial fate modulation of oligodendroglial progenitor cells and adult neural stem cells

The identification and description of resident OPCs in MS lesions, which do not mature into myelinating OLs during remyelination phases [Chang et al., 2002], raised the question on which level cellular differentiation is blocked or how it fails. A better understanding of such limiting mechanisms can most likely be achieved by in-depth investigation of the differentiation patterns and regulatory factors involved. Raff and colleagues describe a developmental clock relevant for early oligodendroglial differentiation. Their research considers whether oligodendroglial progenitors have an “intrinsic clock”, clearly distinguishing between a proliferative cell phase and a following differentiation phase,

driven by cell division counts. A maximum number of cell divisions leads to cell cycle exit of O-2A progenitors resulting in oligodendrocyte differentiation. That mechanism is mediated via growth factors released, for instance, by astrocytes in the optic nerve [Raff et al., 1985]. In addition, thyroid hormone, glucocorticoids and retinoic acid trigger the correct timing of oligodendroglial differentiation [Barres et al., 1994]. Since earlier studies suggested an involvement of the cyclin-dependent kinase inhibitor $p57^{Kip2}$ in the intracellular timing and regulation of cell cycle exit in OLs [Dugas et al., 2007], the modulation of $p57^{Kip2}$ expression may represent an option to control this "intrinsic clock". Intrinsic cell cycle proteins may thereby determine the maximum number of cell divisions, the cell fate and the induction of differentiation rather than solely the presence of extracellular factors and the activation of their corresponding receptors.

In the course of this thesis, a $p57^{Kip2}$-gene suppression approach was applied in primary OPCs and found to accelerate differentiation in OPCs, featuring myelin marker induction as well as morphological maturation [Kremer et al., 2009]. A similar inhibitory effect was also observed in multipotent aNSCs, and in this case it proved even more effective in terms of cell specification than the differentiation process, resulting in a glial fate switch from astrocytic towards oligodendroglial fate [Jadasz et al., 2012b]. Thus, as $p57^{Kip2}$ is not only involved in oligodendrocyte differentiation but also determines cell fates, our observations support the notion that this regulator is probably incorporated in several distinct stages of decision-making to final maturation, irrespective of its function in the "intrinsic clock" hypothesis and of cell cycle exit in proliferative cell phases. Additionally, it was found that OPCs display time windows in which $p57^{Kip2}$ negatively regulates oligodendroglial maturation [Kremer et al., 2009]. In respect of time windows, other proteins have been observed repeatedly as acting in a similar way. For instance, Wnt signalling is known to be important for oligodendroglial differentiation. While it has been shown to drive myelin gene expression of oligodendrocytes, even in different species [Tawk et al., 2011], Tcf4 as a mediator of Wnt signalling is also downregulated in mature oligodendrocytes and plays a role in remyelination [Fancy et al., 2009]. This suggests either multiple and possibly stage-specific effects exerted by this cascade or differential involvement of various pathway members. In this regard it is important to note that a recent publication elucidate differentiation of subependymal zone cells into OLs via live cell imaging tracing and revealed Wnt signalling to be the "fine tuning oligodendrogliogenesis" mechanism which is apparently active in a short time window [Ortega et al., 2013]. Following this reasoning, we hypothesize that stage-specific decision milestones

exist. These decision milestones may be driven by distinct factors and act similarly, nevertheless independent of each other, to the "intrinsic clock" of the cell's development. Moreover, this also emphasizes that the differentiation of OPCs is highly complex and influenced by a number of different factors relating to stage and time specific decisions, the integration of which ultimately determines successful lineage progression or failure.

The regulation of fate decision by $p57^{Kip2}$ as a key regulator became evident when we suppressed and overexpressed $p57^{Kip2}$ in aNSCs and observed lowered and induced GFAP expression levels respectively [Jadasz et al., 2012b]. When $p57^{Kip2}$ expression was downregulated in multipotent aNSCs, it was also observed that these cells clearly initiated oligodendroglial fate decision and differentiation. While these cells could withstand strong astrogliogenic cues, they were not able to progress beyond the OPC stage and generate myelin [Jadasz et al., 2012b]. These observations raise the question of whether the differentiation process is divided into distinct decision milestones, which have to be reached before the subsequent maturation step. If that is the case, already committed OPCs may encounter one last milestone before turning into OLs, achieved via lowered $p57^{Kip2}$ expression levels, for example. By contrast, aNSCs, having the capacity to differentiate into various types of cells and therefore being multipotent, may need to overcome more than one milestone for final lineage-specific maturation.

Having revealed $p57^{Kip2}$ to act as a prominent, nevertheless intrinsic, regulator of glial determination and differentiation, we are confronted by the following key question: How might its expression or activity be modulated from outside the organism? It furthermore remains to be shown which factors are helpful in reaching the milestones and consequently leading to the next.

3.2 External stimulation of oligodendroglial progenitor cells and adult neural stem cells increases the number of oligodendrocytes

Cells of mesodermal origin have a well-known impact on neural development. Mesenchymal stem cells are stromal cells and represent a rich source of secreted stimuli and factors. Since they are embedded in the bone marrow and regulate hematopoietic stem cell biology, they may secrete factors that regulate other stem cells. Rivera

and colleagues found a significant interaction between MSCs and adult neural stem cell differentiation. Here, aNSCs differentiate into oligodendrocytes with the addition of mesenchymal stem cell secreted factors [Rivera et al., 2006]. It was therefore of particular interest to explore to what degree MSC-secreted factors are also able to counteract this conversion. While summarizing these data together with the remyelination potential of aNSCs, OPCs and MSCs [Jadasz et al., 2012a], we began to ask ourselves whether the secretion factor cocktail also modulates predetermined OPC lineage cells. Indeed, we were able to demonstrate that OPCs incubated with this cocktail can act similarly in that such a cocktail can promote the generation of myelin markers accompanied by an accelerated formation of sheaths [Jadasz et al., 2013]. Additionally, we found that oligodendroglial fate is consolidated even under astrocytic (serum-containing) conditions featuring Id down- and Olig2 upregulation [Jadasz et al., 2013]. This observation correlates well with a putative milestone at the oligodendroglial-astroglial (O-A) axis branch point, which recapitulates earlier concepts on the nature of O-2A cells [Raff et al., 1983]. This milestone, triggering oligodendroglial lineage, might be achieved via different pathways, such as Wnt- or anti-BMP signalling or via Id/Olig2 orchestrated homeostasis. Against this background, it is likely that this cocktail acts on several milestones during fate determination and cellular differentiation, since we were able to achieve fully mature oligodendrocytes by external modulation.

Moreover, having described two different ways to influence oligodendroglial differentiation — intrinsic regulation via $p57^{Kip2}$ modulation and extrinsic regulation via addition of a mesenchymal factor cocktail — one might speculate to what degree both mechanisms are connected or influence one another. Thus, in my diploma thesis I was able to demonstrate that $p57^{Kip2}$ has no additional impact on MSC-CM-associated effects in aNSCs [Jadasz, 2009]. However, this does not mean that there is no connection at all and may suggest that all milestones for oligodendroglial differentiation of aNSCs have been reached, likely on account of the secreted cocktail of factors. In this regard it will be of interest to study (post-) transcriptional regulation of $p57^{Kip2}$ expression and activity on contact with mesenchymal stem cell-conditioned media. It will be interesting to discover which signalling tracks are regulated by MSC-CM to determine common functions of both inhibitory and activator processes. Additionally, it remains to be shown whether $p57^{Kip2}$ regulation or function is part of the mesenchymal stem cell mode of action.

3.3 MSC factors involved in oligodendroglial differentiation

It is a well-known fact that MSCs have neuroprotective effects [Karussis et al., 2008]. They produce neurotrophic factors which ameliorate neural cell survival and the brain's regenerative ability [reviewed in Kassis et al., 2011]. Nevertheless, it remains to be shown in detail which factors are involved in the different steps. Ciliary neutrophic factor is known to be produced by MSCs [Chen et al., 2011; Wislet-Gendebien et al., 2004] and to improve remyelination of transplanted OPCs after spinal cord injury [Cao et al., 2010]. However, it does not appear to be involved in MSC mediated differentiation effects [Rivera et al., 2008]. Similarly, we also demonstrated that the decisive MSC-CM component driving the cells towards the oligodendroglial lineage is not hepatocyte growth factor (HGF) [Jadasz et al., 2013], in spite of its proven efficiency in MSC-dependent immunomodulation [Bai et al., 2012]. Furthermore, MSCs are able to migrate [Ji et al., 2004], guided by the chemokine CXCL12 [Stich et al., 2009], on account of the fact that MSCs express its receptor CXCR4 but are also able to secrete CXCL12 themselves [Zhang et al., 2007]. Chemokines such as CXCL12 have been described as having a pro-oligodendrogenic effect via CXCR7 signalling cascade [Göttle et al., 2010]. Given that CXCL12 was initially identified as stromal derived factor-1 (SDF-1), it remains to be shown to what degree stromal and mesenchymal stem cell activities overlap and whether this chemokine is involved in the MSC-dependent determination and/or differentiation effect.

3.4 Preventing astroglial development

The ability of a cell to differentiate into the mature stage requires a specific fate choice, which may reflect the fate decision milestone. Adult NSCs, which reside in special niches of the brain and mainly give rise to new neurons, can become activated as a result of neuroinflammation and direct their fate towards the oligodendroglial lineage [Menn et al., 2006]. This may constitute a decisive milestone in brains affected by MS. Although the transition of stem cells to oligodendrocytes is lost after a certain period in a pathological milieu, such a disease-dependent fate decision is remarkable and deserves closer attention and analysis of underlying signals and mechanisms. Whether

this involves neutralisation of molecules such as BMP-2 and BMP-4 [Gomes et al., 2003; Nakashima et al., 2001; Samanta and Kessler, 2004], or modulation of neurogenic factors and pathways, remains to be shown. However, the $p57^{Kip2}$ suppression mediated effect was revealed to dominate the BMP-driven astrocytic cues. Nevertheless, we know that early OPCs have the potential to direct their fate into astrocytes under the influence of foetal bovine serum (FBS) or BMP-2 and BMP-4 [Gard et al., 1995; Mabie et al., 1997]. Furthermore, the inhibition of BMP and its Smad mediated pathway results in an oligodendroglial fate decision of aNSCs which is driven by the transcription factor Olig2 [Copray et al., 2006; Maire et al., 2009]. When activated, Olig2 also determines this fate decision milestone on demyelination [Fancy et al., 2004]. In this respect it remains to be examined whether $p57^{Kip2}$ also coordinates the behaviour of the less potent oligodendroglial progenitor cells under more specific promoters of the astroglial lineage such as BMPs. It will be interesting to elucidate at what level $p57^{Kip2}$ activity interferes with the BMP pathway.

In addition, recent observations indicate that Wnt signalling is part of the MS pathology-dependent switch, and in this regard it is of interest to note that our preliminary investigations revealed a number of Wnt signalling components regulated in $p57^{Kip2}$ suppressed OPCs. Future studies will be necessary to address exact functional roles in astrogenic differentiation as well as early determination events and to what degree and at which level $p57^{Kip2}$ interferes with Wnt signalling.

3.5 Does $p57^{Kip2}$ control the glial-neuronal axis?

The multipotent capacity of adult neural stem cells results not only in decision milestone attainment between oligodendroglial and astroglial branch point specification, but also at the level of glial versus neuronal cell determination. This glial-neuronal (G-N) axis displays neurogenic differentiation of aNSCs but is also a branch point in decision-making in embryonic stages. For instance, the glial cells missing (Gcm) gene in Drosophila has been described to control G-N fate decision of neural stem cells during embryonic development [Jones et al., 1995]. It is of note that Flici and colleagues showed Gcm to have a decreasing switching potential at late stages of embryogenesis [Flici et al., 2011]. The central question then becomes whether dynamic gene expression is required to change their regulating ability at different decision branch points and at various points in time.

Underlining this speculation, Furutachi and colleagues discuss the role of $p57^{Kip2}$ in adult mice. Here, a short-term (up to 18 days) CreERT2/tamoxifen-mediated depletion of $p57^{Kip2}$ in young adult mice (two to four months old) leads to decreased numbers of astrocytes in the SGZ. Meanwhile, long-term depletion over a period of 24 months, starting one month after birth, does not affect astrogenesis [Furutachi et al., 2013]. It therefore appears that an up- or downregulation of $p57^{Kip2}$ in specific time windows can play an important role in finely tuned developmental changes. Furthermore, loss of $p57^{Kip2}$ during embryonic development triggers neurogenesis [Gui et al., 2007; Joseph et al., 2009]. This finding contrasts with our own studies, in which we do not observe increased neuronal marker expression on suppression of $p57^{Kip2}$ [Jadasz et al., 2012b], in spite of the fact that Joseph and colleagues also describe decreased GFAP levels upon loss of $p57^{Kip2}$ [Joseph et al., 2009]. With regard to the remark "species does matter" [Steffenhagen et al., 2011], such discrepancies may derive from the fact that we used stem cells derived from rats, while other studies describe neural cell changes in mice cells [Furutachi et al., 2013; Gui et al., 2007; Joseph et al., 2009]. Age does matter too. We used three-month-old rat aNSCs in contrast to other researchers, who described the role of $p57^{Kip2}$ in embryonic cells [Gui et al., 2007; Joseph et al., 2009]. One explanation might also be that the G-N axis milestone, which is regulated by $p57^{Kip2}$, must be determined before the glial-glial axis (between oligodendroglial and astroglial cells) is set — a notion that is supported by the observation that all $p57^{Kip2}$-dependent neuronal decisions are taken on embryonic or early adult neural stem cells. This explanation might consequently support a lowered potential of stem cells, since aNSCs may partially lose their neurogenic potency as a result of epigenetic changes or intrinsic differences during development.

In light of the neurogenic potential of $p57^{Kip2}$, Ye and colleagues showed an interaction with another Cip/Kip family member during embryonic neuronal differentiation. Here, $p27^{Kip1}$ expression replaces the expression of $p57^{Kip2}$. In the first step, $p57^{Kip2}$ activates differentiation into neuronal lineage. A then diminished $p57^{Kip2}$ expression followed by an upregulation of $p27^{Kip1}$ lead to cell migration and maintenance of differentiation, while the cells express neuronal marker TuJ1 [Ye et al., 2009], underscoring the hypothesis that $p57^{Kip2}$ plays a key role at the G-N branch point. Others have also described this relationship, which was shown after $p27^{Kip1}$ knockdown leading to enhanced mRNA levels of $p57^{Kip2}$ [Itoh et al., 2007]. $p57^{Kip2}$ may therefore adopt the role of $p27^{Kip1}$, which is not possible in the other direction and as such represents a

unique feature of $p57^{Kip2}$ among Cip/Kip family members.

As both Cip/Kip family members share similar domains [Lee et al., 1995; Sherr and Roberts, 1995], it could be interesting to reveal whether, in spite of their neurogenic potential, the fate switch potential is similar among these proteins. Likewise, it is known that the active division process of type C cells during adult neurogenesis is regulated by cell cycle inhibitor $p27^{Kip1}$ and accelerated by reduced expression [Doetsch et al., 2002]. As we observed $p57^{Kip2}$ protein expression in SVZ-derived aNSCs (Fig. 2.7), it remains to be shown whether it has similar effects on type C cells in that phase.

In conclusion, based on the fact that $p57^{Kip2}$ is involved in fate determination on the O-A axis in adults and also controls embryonic as well as adult neurogenesis, there is evidence of a global key regulation effect of this CDKI. It may be worth elucidating the more plastic regulation of neuronal fate, probably due to differences in cell origin and age. In this regard it remains to be shown whether cells from other brain regions are also fate-modulated by $p57^{Kip2}$ to demonstrate this G-N decision milestone.

3.6 Can we predict the potential of heterogeneous progenitors in the central nervous system?

Adult neural stem cells and progenitors are still insufficiently described, and it is still not clear to what degree subpopulations and overlapping features and identities exist. The current lack of specific markers that can be used to unambiguously identify glial progenitor cells illustrates this limitation. At the moment, we are not able to distinguish between multipotent stem cells generating neurons and other more restricted glial cell types. There is a lack of a clear identification of glial cells and the extent to which different glial cells are responsible for specific developmental steps. Most notably, however, the nature of the cells involved in cell homeostasis is not fully known. For instance, spinal cord injuries show a wide heterogeneity of GFAP-positive astrocytic cell types [Wanner et al., 2013]. Additionally, after brain injury, different astrocytes act differently in the lesion area [Bardehle et al., 2013]. This raises the question of whether these cells are fundamentally different cell types or whether they are the same but possess different potencies resulting in distinct behaviours and reactions. In this regard it is important to note that Bardehle and colleagues claim that a proliferation step distinguishes reactive

from non-reactive astrocytes [Bardehle et al., 2013]. Considering that the multifunctional protein $p57^{Kip2}$ was initially identified as a cell cycle regulator, it is possible to speculate that $p57^{Kip2}$ may regulate reactive versus non-reactive phenotypes via its cell cycle regulatory function. Given that we observed that neither aNSC fate [Jadasz et al., 2012b] nor OPC differentiation [Kremer et al., 2009] depends on $p57^{Kip2}$'s CDKI domain, such a notion could increase the potential impact of this regulator and include further protein domains. Furthermore, it remains to be shown what specifically distinguishes different GFAP-positive cells from each other and whether the $p57^{Kip2}$-dependent glial fate switch potential acts on the global population rather than discriminating different potency stages of multipotent GFAP-positive cells.

Some of these heterogeneity arguments have been adapted by means of conformity to other cell types in the CNS. For instance, Viganò and colleagues propose that white matter and grey matter niches play a crucial role in heterogeneous oligodendrocyte differentiation. Adult OPCs derived from these niches have been transplanted back into either WM or GM. It was shown that WM-derived OPCs have a greater capacity to mature into oligodendrocytes. When transplanted into WM or GM, these cells give rise to myelin-producing OLs, whereas GM-derived OPCs are not as efficient as their WM-derived counterparts. This together indicates that a certain degree of glial heterogeneity exists within the adult OPC pool and that it is related to their location within different areas of the brain [Viganò et al., 2013]. Most importantly, it is currently not known whether oligodendroglial cells derived from multipotent adult NSC are true oligodendrocytes or only oligodendrocyte-like cells; this relates in particular to those cell derivatives that are derived by modulation (forced downregulation) of the intrinsic regulator $p57^{Kip2}$. We showed that transplanted stem cells in both white and grey matter of the spinal cord display a similar switch from astro- to oligodendrocyte features on $p57^{Kip2}$ downregulation [Jadasz et al., 2012b]. Against this background, the question arises as to whether $p57^{Kip2}$-suppressed adult NSC-derived oligodendroglial cells exert a differentiation potential that is similar to either adult GM or WM OPCs of the brain. We additionally ask whether generated OPCs from $p57^{Kip2}$-suppressed adult NSCs can give rise to fully matured oligodendrocytes or to oligodendroglial-like cells only, and to what degree adult NSCs can therefore contribute to glial heterogeneity of the adult brain. A transplantation study into the WM and GM of the brain will therefore show whether these cells can equally mature in white and grey matter and whether these cells behave like GM or WM cells.

3.7 Does $p57^{Kip2}$ assist in reprogramming cells?

The 2012 Nobel Prize was awarded to John Gurdon and Shinya Yamanaka for their discovery of reprogramming adult cells into pluripotent embryonic-like stem cells. These findings reinforce the concept that fate decision is a central aspect of development and differentiation. In the studies of Yamanka, the committed fate of mature cells is reset by means of transcription factor overexpression [Takahashi and Yamanaka, 2006]. Such induced pluripotent stem cells possess naïve embryonic stem cell characteristics [Di Stefano et al., 2010], and they have been shown to be able to differentiate into cardiomyocytes, neuronal and glial cells [reviewed in Liu and Zhang, 2011], suggesting great therapeutic potential [Bellin et al., 2012]. This technique is still undergoing further development, because reprogramming of cells through retroviral vectors driving embryonic transcription factors Oct4, Klf4, c-Myc and Sox2 (OKMS) is still rather inefficient [Takahashi and Yamanaka, 2006]. Some initial improvements were shown on adding or interchanging transcription factors [Liao et al., 2008]. Interestingly, the CDKI protein $p27^{Kip1}$ was found to assist in reprogramming cells into iPSCs by directly regulating Sox2 expression [Li et al., 2012]. The same procedure was described for $p21^{Cip1/Waf1}$ [Marqués-Torrejón et al., 2013], both of which are Cip/Kip family members and are closely related to $p57^{Kip2}$. Such findings suggest that $p57^{Kip2}$ could also favour fate reversal and pluripotency stages by regulating Sox2 expression based on demonstrated co-localization of $p57^{Kip2}$ with Sox2 in the DG and SVZ (Fig. 2.6). Functional studies in cultured cells and *in vivo* will reveal to what degree such a relation exists and whether $p57^{Kip2}$ affects cellular reprogramming.

3.8 Further Research

A major application resulting from our findings could be the development of suitable and stable cell replacement strategies for diseases such as MS. In this regard, directed differentiation and oligodendroglial fate stabilization could be important for endogenous stem and progenitor cells as well as for grafting strategies. As already discussed [Jadasz et al., 2012b], to do this the myelination capacity of transplanted stem cells can be tested in a myelin-devoid background as represented by the shiverer mouse mutant [Dupouey et al., 1979]. Transplantation of either NSCs or OPCs following $p57^{Kip2}$ modulation or

MSC stimulation can then be used to assess terminal differentiation efficiency and the generation of myelin sheaths. Additionally, by empirically examining transplanted cells into particular brain tissues such as WM or GM, we hope to produce a more complete understanding of the heterogeneous potential of different environments.

Furthermore, repairing spinal cord injuries is of great medical importance, but it currently continues to be an unachieved therapeutic goal on account of the inefficient regenerative character of adult CNS remyelination. The NSCs' transition capacity to OPCs is of potential interest for SCI repair, as myelinating glia cells assume an important function in supporting and/or improving regenerative capability. An adopted oligodendroglial progenitor stage of stem cell derivatives is likely to possess modulatory as well as cell replacement properties, which could prove ideal for tissue repair. For instance, aNSCs mediate axonal regeneration in the injured spinal cord [Sandner et al., 2012]. It will therefore be important to discover to what degree the glial fate decision role of $p57^{Kip2}$ in suppressed NSCs can ameliorate the fate switch towards oligodendroglial progenitors under pathological conditions such as the hemisectioned spinal cord. This could represent a primary contribution to future replacement therapies.

Given the slow NSC-OPC transition of aNSCs observed on $p57^{Kip2}$ suppression, genetically modulated cells could have immunomodulatory effects at the lesion site. This hypothesis is based on the work of Martino and Pluchino in 2006. They describe a contact between NSCs and phagocytes after transplantation into SCI lesions. This interaction leads to reduced numbers of macrophages and to the conclusion that inflammatory sites can be modulated by NSCs promoting recovery after injury [Martino and Pluchino, 2006]. In addition, the generation of myelinating glial cells *in situ* could have a strong positive impact on axonal regeneration and stabilize regrown axons, as has recently been shown for unrestricted somatic stem cells (USSCs) from human umbilical cord blood in a spinal lesion paradigm [Schira et al., 2012]. For this purpose we set out to stably modulate aNSCs by means of lentiviral transduction and use them as long-term cell grafts at spinal cord lesion sites. Observing cells and the surrounding injured CNS tissue over a long time period of up to four months will make it possible to study long-term axonal outgrowth, myelin building and immune cell infiltration in response to modulated $p57^{Kip2}$ expression levels.

It will likewise be important to see whether $p57^{Kip2}$ can also modulate fate and differentiation of other stem cell types such as rodent USSCs, MSCs, or human iPSC-derived NPCs and whether such derivates can be used in exogenous cell replacement approaches.

The best way of addressing the *in vivo* relevance of our findings is likely to be by using suitable $p57^{Kip2}$ mouse mutants in which expression can be switched off according to specific stages and cells. Conventional knockout mice for $p57^{Kip2}$ are lethal after birth and therefore do not represent an appropriate tool in gaining further insights into aNSC biology.

3.9 Conclusion

In conclusion, this thesis provides insights into glial fate decision and differentiation of multipotent adult neural stem cells and oligodendroglial progenitor cells which exert different potencies. The lineage progression and modulation of progenitor cells and stem cells was modulated on suppression of $p57^{Kip2}$ as well as by a cocktail of factors secreted by mesenchymal stem cells. We claim that newly generated oligodendroglial cells pass through several decision milestones before achieving maturity while astroglial development is inhibited, and that the individual milestones are modulated dynamically. A detailed knowledge of their nature and composition may foster individually tailored therapeutic approaches for targeted and specific cell replacement as envisaged in the future treatment of demyelinated diseases such as MS.

Appendix A

Appendix — Additional publication

A.1 Lidocaine metabolites inhibit glycine transporter 1: a novel mechanism for the analgesic action of systemic lidocaine?

Robert Werdehausen*, David Kremer*, Timo Brandenburger, Lukas Schlößer, Janusz Joachim Jadasz, Patrick Küry, Inge Bauer, Carmen Aragón, Volker Eulenburg, and Henning Hermanns

Abstract

Background: Lidocaine exerts antinociceptive effects when applied systemically. The mechanisms are not fully understood but glycinergic mechanisms might be involved. The synaptic glycine concentration is controlled by glycine transporters. Whereas neurons express two types of glycine transporters, astrocytes specifically express glycine transporter 1 (GlyT1). This study focuses on effects of lidocaine and its major metabolites

* These authors contributed equally to this work.

on GlyT1 function.

Methods: The effects of lidocaine and its metabolites mono-ethyl-glycine-xylidide (MEGX), glycinexylidide, and N-ethylglycine on GlyT1 function were investigated in uptake experiments with [14C]-labeled glycine in primary rat astrocytes. Furthermore, the effect of lidocaine and its metabolites on glycine-induced currents were investigated in GlyT1-expressing Xenopus laevis oocytes.

Results: Lidocaine reduced glycine uptake only at toxic concentrations. The metabolites MEGX, glycinexylidide, and N-ethylglycine, however, significantly reduced glycine uptake ($P<0.05$). Inhibition of glycine uptake by a combination of lidocaine with its metabolites at a clinically relevant concentration was diminished with increasing extracellular glycine concentrations. Detailed analysis revealed that MEGX inhibits GlyT1 function ($P<0.05$), whereas N-ethylglycine was identified as an alternative GlyT1 substrate (EC_{50} = 55 µM).

Conclusions: Although lidocaine does not function directly on GlyT1, its metabolites MEGX and glycinexylidide were shown to inhibit GlyT1-mediated glycine uptake by at least two different mechanisms. Whereas glycinexylidide was demonstrated to be an alternative GlyT1 substrate, MEGX was shown to inhibit GlyT1 activity in both primary astrocytes and in GlyT1-expressing Xenopus laevis oocytes at clinically relevant concentrations. These findings provide new insights into the possible mechanisms for the antinociceptive effect of systemic lidocaine.

Approximated total share of contribution: 15%

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ImpactFactor: 5.3

Contribution on experimental design, realization and publication

Tissue preparation and stainings were performed by Janusz Joachim Jadasz. The manuscript was reviewed, amended and approved by all co-authors.

Link to the publication

`http://journals.lww.com/anesthesiology/pages/articleviewer.aspx?year=2012&issue=01000&article=00027&type=abstract`

Appendix B

Appendix — Poster presentations

1. David Kremer, André Heinen, Janusz Joachim Jadasz, Peter Göttle, Kristin Zimmermann, Philip Zickler, Sebastian Jander, Hans-Peter Hartung, and Patrick Küry (2009). A novel intrinsic inhibitor of oligodendroglial differentiation and maturation, *ECTRIMS*, Düsseldorf

2. Janusz Joachim Jadasz, Agens Taubert, Francisco J. Rivera, Mahesh Kandasamy, Ludwig Aigner, Hans-Peter Hartung, and Patrick Küry (2009). A novel regulator of neural stem cell determination/differentiation, *ECTRIMS*, Düsseldorf

3. Janusz Joachim Jadasz, Agens Taubert, Francisco J. Rivera, Mahesh Kandasamy, Beatrice Sandner, Norbert Weidner, Ludwig Aigner, Hans-Peter Hartung, and Patrick Küry (2011). A novel molecular switch of astroglial versus oligodendroglial cell fate determination relevant for Multiple Sclerosis related remyelination, *Current Topics in Myelin Research*, Kassel

4. Janusz Joachim Jadasz, Agens Taubert, Francisco J. Rivera, Mahesh Kandasamy, Beatrice Sandner, Norbert Weidner, Ludwig Aigner, Hans-Peter Hartung, and Patrick Küry (2011). p57^{Kip2} is an intrinsic regulator of adult neural stem cell glial fate decision, *10th European Meeting on Glial Cells in Health and Disease*, Prague

5. Janusz Joachim Jadasz, Agens Taubert, Francisco J. Rivera, Mahesh Kandasamy, Beatrice Sandner, Norbert Weidner, Orhan Aktas, Ludwig Aigner, Hans-Peter Hartung, and Patrick Küry (2012). p57^{Kip2} regulates glial fate decision in adult neural stem cells, *Gordon Research Conference — Myelin*, Lucca/Barga Tusceny

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Addendum

For copyright reasons, this published version of the cumulative thesis document does not contain reprints of all articles according to "§ 12 (2) Promotionsordnung from 12.6.2013". Instead, only the abstracts is added along with the the link to the publication. The following article is not included as a reprint.

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Declaration of Authorship

Ich, Janusz Joachim Jadasz, versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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Date: November, 2013