AUS DEM LEHRSTUHL
FÜR NEUROLGIE
PROF. DR. MED. ULRICH BOGDAHN
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

The species specific effects of Mesenchymal stem cell conditioned medium on proliferating mouse and rat adult neural stem/ progenitor cells

Inaugural - Dissertation
zur Erlangung eines Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Franz – Xaver Dechant
Regensburg, 2011

AUS DEM LEHRSTUHL
FÜR NEUROLGIE
PROF. DR. MED. ULRICH BOGDAHN
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

The species specific effects of Mesenchymal stem cell conditioned medium on proliferating mouse and rat adult neural stem/ progenitor cells

Inaugural - Dissertation
zur Erlangung eines Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Franz – Xaver Dechant
Regensburg, Anno Domini 2011

Dekan:	Prof. Dr. Dr. Torsten Reichert
1. Berichterstatter	Prof. Dr. rer. nat. Ludwig Aigner
2. Berichterstatter	PD Dr. med. Martin Proeschodt
Tag der Mündlichen Prüfung:	29. März 2012

IN ACKNOLEDGEMENT OF MY GREAT GRATITUDE DEDICATED TO REBECCA MAYR.

οὐ γάρ ἐστιν κρυπτὸν ὃ οὐ φανερὸν γενήσεται οὐδὲ ἀπόκρυφον ὃ οὐ μὴ γνωσθῇ καὶ εἰς φανερὸν ἔλθῃ.

Lk 8, 17

FOR THERE IS NOTHING HIDDEN THAT WILL NOT BE DISCLOSED, AND NOTHING CONCEALED THAT WILL NOT BE KNOWN OR BROUGHT OUT INTO THE OPEN.
LUKE 8, 17

Content

Figure legend

Table legend

List of abbreviations

α-MEM	Alpha Modified Eagles Medium
A2B5	Progenitor marker
B-27 supplement	serum substitute to support the cultures of neurons at low or high cell densities in both short and long term cultures
BMP	bone morphogenetic protein
BrdU	Bromo-deoxyuridine
cDNA	Complementary DNA
CNPase	Oligodendrocytes Marker
CNS	Central nervous system
CNTF	Ciliary neurothropic factor
CO_2	Carbon dioxide
DAPI	4', 6'-diamidino-2-phenylindole dihydrochloride hydrate
DCs	Dendritic cells
DCX	Doublecortin
DG	Dentate gyrus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleotide acid
EGF	Epidermal growth factor
FACS	Fluorescence-Activated Cell Sorter
FGF	Basic fibroblast growth factor
FSGB	Fish Skin Gelatin Buffer
FOX-O	Forkhead box, class O)
GAPDH	Glycerinaldehyde-3-Phosphate-Dehydrogenase
GFAP	Glial Acidic Fibrilary Protein
GFW	growth factor withdrawal
h	hour
HC	Hippocampus
HSC	Haematopoietic stem cell
Id	Inhibitor of differentiation
i.e.	id est
IGF-1	Insulin-like growth factor

IL-6	Interleukin 6
LDH	Lactate dehydrogenase
LV	Lateral ventricle
M	mol
MAP2ab	Microtubule-associated Protein 2a + 2b
MBP	Myelin Basic Protein
MEM	Modified Eagles Medium
$MgSO_4$	Magnesium sulphate
mNSph	Mouse Neurospheres
mRNA	messenger RNA
MSC	Mesenchymal stem cell
MSC-CM	Mesenchymal stem cell conditioned medium
MS	Multiple sclerosis
NPC	Neural progenitor cell
NSC	Neural stem cell
NSPCs	Adult derived neural stem/progenitor cells
NSph	Neurospheres
O4	Oligodendrocytes Marker
Olig-1/-2	Oligodendrocytes transcription factor 1/2
OPC	Oligodendrocytes Progenitor Cell
OPr	Oligodendrogenic program
PBS	Phosphate buffered NaCl-solution
PCR	Polymerase chain reaction
PDGF-A	Platelet-Derived Growth Factor A
PFA	Paraform aldehyde
PI	Propidium Iodide
PI3K-Akt	Phosphatidylinositol 3-kinase-Akt
PNS	peripheral nervous system
PSA NCAM	Poly-Sialated Neural Cell Adhesion Molecule
rNSph	Rat Neurospheres
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SMI94	Antibody against Myelin Basic Protein

Sox2	SRY (sex determining region Y)-box 2
TGF-beta1	transforming growth factor type-beta-1
SVZ	Subventricular zone

Zusammenfassung

In den verschiedenen Geweben des erwachsenen Organismus kommen unterschiedliche Arten von Stammzellen vor. Dazu gehören neben Mesenchymalen Stammzellen aus dem Knochenmark (MSCs), Neuronale Stamm und Progenitor Zellen (NSPCs) aus verschiedenen Regionen des Zentralen Nervensystems (CNS). Diese NSPCs können in der Form von Neurospheres (NSph) als *in vitro* Modell für die adulte Neuro- und Gliogenese kultiviert werden.

Auch wenn die zu Grunde liegenden Mechanismen ungeklärt sind, zeigten *in vitro* Experimente, dass von MSCs produzierte lösliche Faktoren in speziesspezifischer Weise neuroprotektiv und neuroregenerativ wirken. So lassen diese löslichen Faktoren differenzierende NSphs der Ratte zu Oligodendrozyten ausreifen (Rivera et al., 2006). Im Gegensatz dazu führen diese Mediatoren zur Astro- und Neurogenese differenzierender NSphs bei der Maus (Bai et al., 2007). Diese Linienspezifizierung der Zellidentität von Stammzellen tritt allerdings nicht nur während der Differenzierungsphase, sondern bereits im Proliferationsstadium auf. Vor diesem Hintergrund untersucht die vorliegende Doktorarbeit ob lösliche Mediatoren von MSCs fähig sind die Zellidentität proliferierender NSphs zu primen oder sie zu verändern. Im Weiteren geht diese Arbeit der Frage nach, ob diese Effekte in speziesspezifischer Weise auftreten.

Um diese Fragenstellungen zu klären, wurden proliferierende NSphs von Maus und Ratte mit Nährmedium inkubiert, womit vorher MSCs inkubiert worden waren (MSC-CM). Im Folgenden wurden die Auswirkungen von MSC-CM, welches die lösliche Faktoren der MSCs enthält, auf die Zellmorphologie, die Proliferationsrate, die zellspezifische Markerexpression, die Reaktion auf Wachstumsfaktorentzug (GFW) und die Expression von neuronalen, astroglialen und oligodendroglialen Markern untersucht.

Während MSC-CM die Proliferationsrate von rNSphs unbeeinflusst ließ, führte es zur Adhäsion der rNSphs und zur Bildung von zellulären Fortsätzen. Das Progenitormarkerprofil der rNSphs war dabei nicht verändert, wohingegen die Ausreifung zu MBP-positiven Oligodendrozyten durch MSC-CM gesteigert wurde. In diesem Zusammenhang zeigte sich außerdem, dass der anti-oligodendrogene Transkriptionsfaktor inhi-

bitor of differentiation 2 (Id2) reduziert war, während der pro-oligodendrogene Transkriptionsfaktor Olig2 erhöht war.

Im Gegensatz zu diesen Ergebnissen zeigte die Behandlung von mNSphs mit MSC-CM eine Reduktion der Proliferationsrate und eine Abnahme der absoluten Zellzahl. Gleichzeitig führte die Behandlung mit MSC-CM zu einer Erhöhung der Laktatdehydrogenasespiegel, verbunden mit der Annahme, dass die im MSC-CM gelösten Faktoren zum Zelltod der mNSphs durch Apoptose oder Nekrose führen. Im Gegensatz zu rNSphs reiften proliferierende mNSphs nicht zu Oligodendrozyten aus. Allerdings fand sich eine Erhöhung von astroglialen Markern ebenso wie eine Erhöhung von Stammzellmarkern. Gleichzeitig zeigte sich eine Erhöhung der Selbsterneuerungsmarker Bmi-1 und Sox-2, was auf potentielle Stammzellen Rückschlüsse ziehen lässt. Auch wenn diese Daten vermuten lassen, dass es sich hierbei um Stammzellen handeln könnte, reichen diese Ergebnisse für einen endgültigen Beweis des Stammzellcharakters nicht aus.
Die in dieser Doktorarbeit beschriebene Möglichkeit in die Biologie proliferierender NSPCs einzugreifen, spielt für die Entwicklung neuer medizinischer Therapien eine große Rolle. Insbesondere die Bedeutung für die Bereiche der Remyelinisation und der autologen Zelltherapie werden durch diese Experimente unterstrichen.

Abstract

Adult stem cells reside in different tissues and organs of the adult organism. Among these cells are Mesenchymal stem cells (MSCs) and neural stem and progenitor cells (NSPCs).

NSPCs can be obtained from different regions of the central nervous system (CNS) and can be expanded for several passages as neurospheres (NSph) *in vitro*. These NSph cultures are considered as *in vitro* model for NSPCs neurogenesis and gliogenesis.

MSCs, which are located in the bone marrow display neuroprotective, as well as neuroregenerative effects on the adult CNS. Although the underlying mechanisms are largely unidentified, *in vitro* experiments revealed that soluble factors derived from MSCs affect the adult NSPCs biology in a species-dependent manner. Thus, in a previous study Rivera et al. demonstrated that soluble factors derived from MSCs induce oligodendrogenesis in differentiating adult rNSPCs (Rivera et al., 2006), whereas MSCs induce astro-/ neurogenesis in differentiating adult mNSPCs (Bai et al., 2007). However, since lineage specification also occurs in proliferating progenitors and not only during differentiation, this thesis aims to investigate if soluble factors derived from MSCs are able to prime and / or change the fate of proliferating NSphs. Furthermore this dissertation proposes to confirm whether these effects of MSC-CM occur in a species dependent manner. Therefore, the effects of conditioned medium derived from MSCs (MSC-CM) on adult rat and mouse derived NSph cell morphology, proliferation, cell-specific marker expression profile, response to growth factor withdrawal (GFW), and the expression of neural, astroglial and oligodendroglial fate determinants were analyzed. While MSC-CM did not affect the proliferation rate, it induced the formation of cellular processes and the adherence of proliferating rNSphs. Even the expression profile of progenitor and precursor markers was not affected by MSC-CM in rNSPCs, the formation of MBP positive oligodendrocytes after GFW was boosted by MSC-CM pre-treatment. Moreover, in proliferating rNSphs, MSC-CM reduced the anti-oligodendrogenic determinant inhibitor of differentiation-2 (Id2), thus increasing the relative proportion of the pro-oligodendrogenic factor Olig2 expression.

However, mNSphs displayed a reduction in the proliferating rate and a reduction of the absolute cell number. The increased levels of lactate dehydrogenase (LDH) after treatment with MSC-CM indicate that MSCs induced cell death through apoptosis or necrosis in proliferating mNSphs.

Interestingly MSC-CM did not prime mNSPCs towards an oligodendrogenic fate decision, but it let increase the astroglial / and stem cell marker levels. In the same manner MSC-CM the self-renewal determinants Bmi1 and Sox-2 were increased after MSC-CM treatment. Although these data suggest a stem-cell character of these remaining mNSphs it is not possible to conclude that they display a full-fully stem cell phenotype.

In summary, soluble factors derived from MSCs prime proliferating rNSPCs towards oligodendrogenic fate, whereas it looks like that MSC-CM could shift mNSPCs back to a stem cell phenotype. The present findings underscore the potential use of MSCs in cell therapies like for example for remyelination or autologous cell therapy.

I. Introduction

I.1 Nervous system: Functions and main cell types

By definition the nervous system is the sum of all neural tissues of the body and it has three main functions: receiving information from the sensory and the intrinsic system, integrating this information and responding with behavioural, cognitive and motoric output.

For the practical use the nervous system is divided in two main parts: the central nervous system (CNS), which comprises brain and spinal cord, and the peripheral nervous system (PNS), which is composed of the somatic and the visceral nerve system. While the PNS is characterized by receiving input and mediating output, the CNS is responsible for processing the information in contrast.

The nervous system mainly consists of two specialized cell types, namely neurons and glia cells. Neurons control and integrate all functions of the body. This means that neurons receive, integrate and send a signal to a target cell electrochemically.

The second part of the nervous system is the glia. The term glia or “nerve glue” was coined in 1859 by Rudolph Virchow, who interpreted neuroglia as an inactive “connective tissue” holding the neurons together. Today it is well accepted that glia cells full-fill multiple functions and are no inactive connective tissue. In general glia cells support the physiology and the function of neurons by surrounding them, isolating them from each other, supplying them with oxygen and nutrients and the detoxification of the environment (Eroglu et Barres, 2010).

These versatile duties allow to assume that the glia consist of several cell types. The glia can be divided into two main categories: macro- and microglia.

Astrocytes, oligodendrocytes, Schwann-cells and the cells of the ependyme compose the macroglia, which is derived from the embryonic ectoderm.

As their name suggests, astrocytes are star-shaped, process-bearing cells distributing throughout the CNS. Astrocytes represent the most abundant type of the glial cells and full-fill various functions. They form the glia limitans (Squire., 2008), assure the K^+ and H^+ homeostasis, store glycogen and yield glucose to the neurons (Jayakumar et Norenberg, 2010; Schousboe et al., 2010). Astrocytes have common unique cytological and immunological properties that make them easy to identify, in-

cluding their star shape, their feeds on capillaries to build the blood brain barrier (glia limitans), and a unique population of large bundles of intermediate filaments (Kandel, 2000). These filaments are composed of an astroglial-specific protein commonly referred to as glial fibrillary acid protein (GFAP) (Welsch 2005).

Further tasks of the macroglia are achieved by a second kind of glial cells, named oligodendrocytes. These cells are smaller then astrocytes and don't exhibit GFAP-filaments. The main abandonment of oligodendrocytes is the fabrication of myelin for the CNS. Myelin itself consists of a single sheet of oligodendroglial plasma membrane and builds the myelin sheath. In contrast to the CNS the myelin sheaths of the PNS are built by the Schwann- cells. The myelin sheaths have a high electrical resistance and insolate the axons.

An additional type of macroglial cells are the cells of the ependyme. These cells are facing the walls of the ventricle system and the central canal of the spinal cord. They develop from the embryonic neural epithelium and produce the liquor.

The second part of the neural glia is the microglia. Microglia is most likely derived from bone marrow monocytes that enter the brain during development (Kaur et al., 2001). In addition to these bone-marrow derived monocytes, mesodermal pial elements, neural epidermal cells and capillary associated pericytes are told to be the source of microglia (Napoli et Neumann, 2009). Microglia cells have been termed "the CNS macrophages" and they function as the resident representatives of the immune system in the brain (Navascués et al., 2000).

I.2 Stem cells and progenitor cells

A general definition of the term "stem cell" is the following: Stem cells are undifferentiated, primitive and clonogenic cells which are capable of both self-renewal and multipotency (Temple, 2006). Self-renewal means the almost unlimited capacity to proliferate and thereby the production of identical cells, while multipotency describes the ability to differentiate into a variety of specialized mature cell types (Morrison et al., 1997; Weissman, 2000). Stem cells are found in almost all multicellular organisms and due to their special capacities they are responsible for the development and regeneration of tissue and organ systems.

The differentiation of a stem cell to a mature cell takes place along several stages. Thus, the stem cell gives rise to various types of progenitor and precursor cells. Among experts the terms progenitor and precursor cell are not used consistent. For this reason the following definition of progenitor and precursor cells is used in this thesis: progenitor is used to describe any dividing cell that can generate differentiated progeny, whether or not it can self-renew. In contrast the term precursor is used to describe a cell that is committed to a specific fate. These progenitors and precursors finally differentiate into specific mature cells.

During the lifetime of an organism, tissues must be renewed several thousand times. Consequently, stem cells in the basal layer of the accordant tissue must divide for several times to produce new full differentiated mature cells for the accordant tissue. This process can be maintained only if the stem cell population is self-renewing. There are two ways of proliferation to ensure the capacity of self-renewal: symmetric and asymmetric division. Symmetric cell division means the genesis of two identic daughter cells, with the same fate and the same characteristics, whereas asymmetric cell division stands for the generation of one daughter cell that is a new stem cell, like the original cell and a second, different cell type with limited self-renewing potential and increasing commitment for a particular lineage (fate commitment) (figure 1).

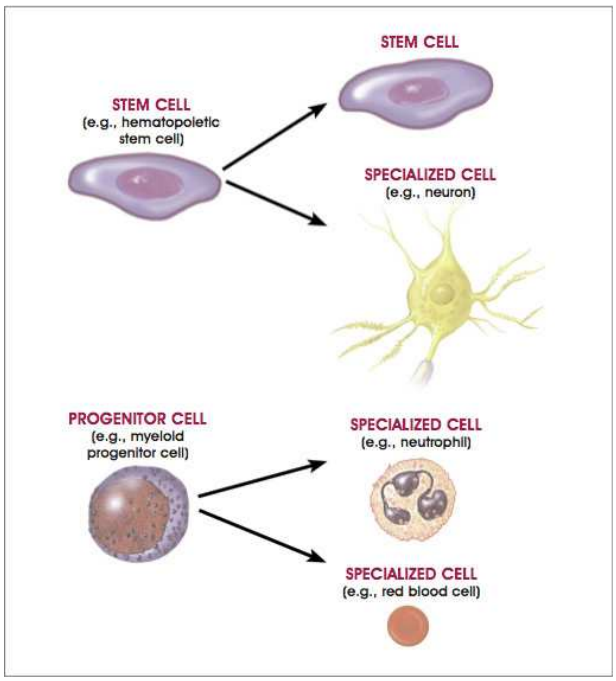


Figure 1. Different characters of stem cells and progenitor/precursor cells.
A stem cell is an immature cell that has the potential to self-renew and to develop into specialized cell types. A stem cell can both produce an additional stem cell with the same characteristics (self-renewal) or the stem cell is able to develop into a mature, fully-differentiated cell, e.g. a neuron. A precursor cell is still capable to proliferate, however with limited self-renewal potential. Here a myeloid progenitor cell is shown, producing to specialized cell types (a neutrophil and a red blood cell) (Figure extracted from NIH Resources. www.ncbi.nih.gov).

I.3. Adult stem cells

I.3.1 Concept and biology

An adult stem cell is an undifferentiated cell, found among an adult tissue or organ that can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found (Basak and Taylor, 2009). Until now several types of stem cells have been described, like haematopoietic stem cells (HSC), neural stem cells (NSC), mesenchymal stem cells (MSC) and endothelial progenitor cells (Pontikoglou et al., 2008).

For many years, the general belief was that tissue-residing, multipotent adult stem cells were developmentally restricted only to differentiate into cell lineages of the specified tissue where they reside, because in contrast to embryonic stem cells, adult stem cells are multipotent but not totipotent.

This traditional view of adult stem cells being restricted in their fate to a specific organ or tissue has challenged in the past few years. In different experimental setups, it has been demonstrated that under certain experimental conditions adult stem cell may loose their tissue or germ layer-specific phenotypes and become reprogrammed to transdifferentiate into cells of other germ layers and tissues (Krause et al., 2001; Woodbury et al., 2002). This term transdifferentiation is commonly used to describe the plastic ability of adult stem cells to differentiate into cell lineages of tissues different from the one in which the somatic stem cell resides and into cells originating from other germ layers.

Although isolated instances of transdifferentiation have been observed in some vertebrate species, whether this phenomenon actually occurs in humans is under debate by the scientific community. Instead of transdifferentiation, the observed instances may involve fusion of a donor cell with a recipient cell equal *in vivo* (Vassilpoulos et al., 2003; Weimann et al., 2003; Weinmann et al., 2003; Alvarez-Dolado et al., 2003) and *in vitro* (Terada et al., 2002; Ying et al., 2002). Another possibility is that transplanted stem cells are secreting factors that encourage the recipient's own stem cells to begin the repair process. Even when transdifferentiation has been detected, only a very small percentage of cells undergo the process (Krabbe et al., 2005).

I.3.2 Bone marrow derived mesenchymal stem cells (MSCs)

The adult bone marrow is a potential rich source of stem cells and it contains two types of prototypical multipotent stem cell populations: haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSC). Both HSCs and MSCs are of mesoderm origin and can be distinguished through several adhesion molecules, extracellular matrix proteins, cytokines and growth factor receptors (Bobis et al., 2001).
MSCs act presumably through paracrine mechanisms as stromal cells to regulate the activity and fate of the HSCs in the bone marrow (Mingell et al., 2001). These paracrine effects of MSCs are not restricted to the bone marrow niche, as, for example MSCs transplantation promoted adult NSPCs proliferation and differentiation in an animal model of stroke (Zhang et al., 2004). The effects of these paracrine, soluble factors of MSCs on NSPCs in *in vitro* models will be discussed in later chapters (f.e. I.5).

Interestingly an increasing amount of researches showed that MSCs are multipotent adult progenitor cells, which have the capacity to trans-differentiate into cells with mesodermal, neuroectodermal and endodermal characteristics *in vivo* and *in vitro*. Although an increasing amount of studies has reported that transdifferentiation of MSCs into neurons and glia cells *in vivo* and *in vitro* could supply the repair of damaged brain areas (f.e. Dharmasoroja et al. 2009) the results are controversially discussed. For *in vivo* experiments, Azizi et al. transplanted MSCs directly in the striatum. Interestingly after three months cells had migrated to the cortex (Azizi et al., 1998). In another study, MSCs, placed into the mouse lateral ventricle (LV) were later detected in cerebellum, HC molecular layer and olfactory bulb. Surprisingly, transplanted MSCs expressed markers specific for astrocytes and neuronal lineage. After MSCs were placed into a CNS trauma, stroke or Parkinson mouse model, transplanted cells were found to express mature astrocyte- or neuronal-specific markers (Kopen et al., 1999; Mahmood et al., 2001; Li et al., 2001; Li et al., 2000). However, follow-up studies revealed the possibility of fusion events between transplanted stem/progenitors cells with endogenous differentiated cells (Kemp et al., 2010; Alvarez-Dolado et al., 2003; Terada et al., 2002).
In vitro studies, which indicate the transdifferentiation of MSCs must be interpreted carefully, too. A huge number of studies showed that the induction of neural genes in MSCs could be achieved through stimulation with non-physiological substances such

as beta mercaptoethanol, dimethylsulfoxide, hydroxyanisole and butylated hydroxy-toluene, etc. (Deng et al., 2001; Munoz-Elias et al., 2003; Padovan et al., 2003; Rismanchi et al., 2003; Sanchez-Ramos et al., 2000; Woodbury et al., 2002; Woodbury et al., 2000; Krabbe et al., 2005; Parr et al., 2008). The criteria to assess the neural differentiation properties of these compounds were based on the appearance of cells exhibiting a typical neural-like morphology and/or the expression of distinctive neural-specific genes.

However, further studies have recommended caution in the interpretation of results assessing the neural differentiation properties induced by non-physiological compounds, since a disruption of the actin cytoskeleton may facilitate the outcome of neurite-resembling processes (Neuhuber et al, 2004). When cells were treated with cytocalasin-D or latrunculin-A (to disrupt the F-actin network), a neuronal-like morphology was acquired, similar to that obtained with neuronal induction media. Moreover, in a study by Lu and co workers, it was demonstrated that morphological changes and increases in immunolabeling for certain neural markers upon “neural chemical induction” of MSCs are likely the result of cellular toxicity, cell shrinkage, and changes in the cytoskeleton and do not represent a true neuronal differentiation (Lu et al., 2004).

I.3.3 Adult neurogenesis and gliogenesis

How the diverse types of neurons and glia are generated by stem and progenitor cells during CNS development and in the adulthood is one of the most enthralling biological questions. For many years it was believed that the adult CNS is incapable of regeneration and that neurogenesis takes place only during embryonic development. Now it is commonly accepted that the adult mammalian brain is not simply a static postmitotic organ (Seaberg et al., 2003; Taupin et al., 2002; Momma et al., 2000). The process of the generation of new neurons from stem cells and progenitors (neurogenesis), which includes proliferation and fate determination as well as differentiation, maturation and final integration into neural circuits (Ming and Song, 2005) is still detectable during adulthood. Since neural stem cells, the source of new neurons were first isolated by Reynold and Weiss from the SVZ of rodents in 1992 and by Kukekov 1999 from humans, the cells attract more and more attention of the researchers.

Interestingly multipotent cells can be isolated from different regions of the mammalian CNS such as the spinal cord and parenchyma of the adult brain throughout the rostrocaudal axis (Johansson et al., 1999, Tend et al., 2002). These data indicate that neurogenesis takes place in several areas of the brain, but not everywhere. To understand the possibilities and the processes of adult neurogenesis, it is necessary to take a look on the areas where adult CNS neurogenesis occurs, named neural stem cell niches. A stem cell niche is a constellation of intrinsic and extrinsic cellular mechanisms, i.e. signalling molecules like growth factors, cytokines or neurotransmitters and ion concentrations, structure of the extracellular matrix and many others, regulating the balance of self renewal and differentiation in stem cells (Moore & Lemischka, 2006). The most interesting neural stem cell niches for research are the SVZ around the LV and the DG of the HC. The hippocampal stem cell niche is located along a thin strip of cells between the hilar region and the granule layer, referred to as the SGZ (Kempermann et al., 2008). It looks like that the adult neural stem cells of the SVZ and the SGZ have a temporal restriction in potency. These cells can generate neurons, astrocytes and oligodendrocytes, but the types of neurons and glia generated may be limited. It has been demonstrated that the adult NSCs of the SVZ divide and differentiate into neural precursors, which migrate via the rostral migratory stream into the olfactory bulb, where they functionally integrate and differentiate into granule- and periglomerular neurons (Doetsch & Scharff, 2001; Carleton et al., 2003). On the contrary the hippocampal stem cells of the SGZ of the DG divide along the border of the hilus locally and give rise to neural precursors, which migrate and integrate into the granule layer of the HC (Cameron et al., 1998).

Furthermore it seems that neural stems of the SVZ and the SGZ cells may be capable of producing cells for other tissues via transdifferentiation. In 1999, Bjornson et al. demonstrated that clonally-derived adult neural stem cells can give rise to haematopoietic cells, when they were injected into sublethally irradiated adult mice. Thereafter it was shown that adult neural stem cells possess the ability to differentiate into skeletal-muscle, both *in vivo* and *in vitro* (Galli et al. 2000a).

Although it has been well established that neurogenesis continues in the adult CNS the fate and the role of these stem cell populations placed in the different stem cell niches remains unclear. Retrograde tracing studies have shown that the newly generated neuronal cells extend axons (Standfield et al., 1988; Palmer et al, 1999), receive synaptic input (Markakis et al., 2004) and participate in functional synaptic cir-

cuitry (Carlen et al., 2002). In addition, it has been hypothesized that these cells have diverse functions such as memory (Feng et al., 2001), learning (Kempermann et al., 2002), and cell replacement (Kokaia et al., 2003). For example there is now growing evidence that injury or disease lead to elevated levels of neurogenesis and cell survival. Ischemic insults have been shown to trigger neurogenesis from neural stem or progenitor cells in the SVZ of the LV, the DG of the HC, and even in the spinal cord (Kokaia et al., 2003).

It goes without saying that neurogenesis is a well orchestrated program and must be regulated precise. Bmi-1, Sox2 and FoxO3a are characterized more in detail in the following, as they are the most important cue transcription factors for this thesis, to regulate the activity of NSC (figure 2). Bmi-1 is necessary for NSC self-renewal and that it represses the cell cycle inhibitors p16, p19 and p21 (Fasano et al., 2008, Molofsky et al., 2003; Molofsky et al., 2005). Foxg1 is a forkhead transcription factor that is essential for forebrain development. Loss of Foxg1 in developing cortical cells inhibits cell proliferation (Shen et al., 2006). Moreover it is known that Foxg1 inhibits p21 levels to stimulate progenitor cell division and acts in part via suppression of p21 (Fasano et al., 2007). Fasano et al., showed moreover that forced expression of Bmi-1 increases the Foxg1 levels specifically in proliferating cells and maintains the NSC population. Thus, self-renewal is reduced in Bmi-1-deficient NSCs derived from Bmi-1 knock out mice, which leads postnatal to depletion of these cells. Furthermore, in the absence of Bmi-1, the cyclin-dependend kinase inhibitor $p16^{Ink4a}$ is unregulated in NSCs leading to a reduced rate of proliferation. Interestingly, restricted neural progenitors from the forebrain proliferate normally in the absence of Bmi-1. That means, while self-renewal of NSCs depends on Bmi-1, proliferation of restricted progenitors is independent of Bmi-1 (Molowsky et al., 2003). In a lot of other studies the crucial role of Sox-2 for the stem cell maintenance in developing and adult brains is demonstrated (Qu and Shi, 2009). Inhibition of Sox-2 expression results in premature neuronal differentiation, whereas consecutive expression of Sox-2 maintains neural progenitor characteristics and inhibits differentiation (Bylund et al., 2003; Graham et al., 2003). In addition, over expression of Sox-2 leads to up regulation of Notch and following Hes5 in neural progenitors (Bani-Yaghoub et al., 2006). With a comparable importance like Bmi-1 and Sox2, pax6 directly regulates genes, controlling the balance between neocortical stem cell maintenance, neurogenesis and the production of basal progenitors in a dose-dependent manner. Increasing pax6-leves drives basal

progenitor cells genesis from cortical stem cells. These basal progenitor cells undergo a limited number of mitotic divisions to generate neurons (Sansom et al., 2009). Pax6 further interacts with three other regulators of the neurogenesis: Neurog2, Ascl1 and Hes1.

A further important role in neurogenesis play the FOX-O proteins (Forkhead box, class O), a subfamily of Forkhead transcription factors. FOX-O is found in the whole body, playing an important role in the energy metabolism, the proliferation, differentiation and survival of stem cells, as well as in many other cellular processes, including the apoptosis and DNA repair. These effects are achieved by the induction or suppression of target genes. Initially, FOX-O1, FOX-O3a and FOX-O4 were identified in fusion genes from chromosomal translocations in human soft-tissue tumours and leukemia. In the neural tissues, such as HC, cortex and cerebellum mostly FOX-O3a is expressed (Maiese et al. 2008). FOX-O proteins are negatively regulated by the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) signalling pathway, which is activated by growth factors and cytokines, like Insulin and IGF-1 (Birkenkamp and Coffer, 2003; Furukawa-Hibi et al. 2005). The constitutive activation of this signalling pathway leads to the development of tumours both through deregulation of cell-cycle progression and through an increase in cellular resistance to proapoptotic signals. The activation of the PI3K-Akt signalling pathway appears to be required for the entry of quiescent cells into the cell cycle through the suppression of the FOX-O proteins. The sustained activity of FOX-O results in up regulation of the expression of $p27^{Kip1}$, p130 and cyclin G2, as well as inhibition of the expression of cyclins D and D2, thereby ensuring maintenance of the quiescent state. (Furukawa-Hibi et al., 2005). Confluent to these conclusions the elimination of FOX-O3a promotes long-term survival of neuroblasts and sustains neurogenesis (Siegrist et al., 2011; Chiacchiera and Simone, 2010) (figure 2).

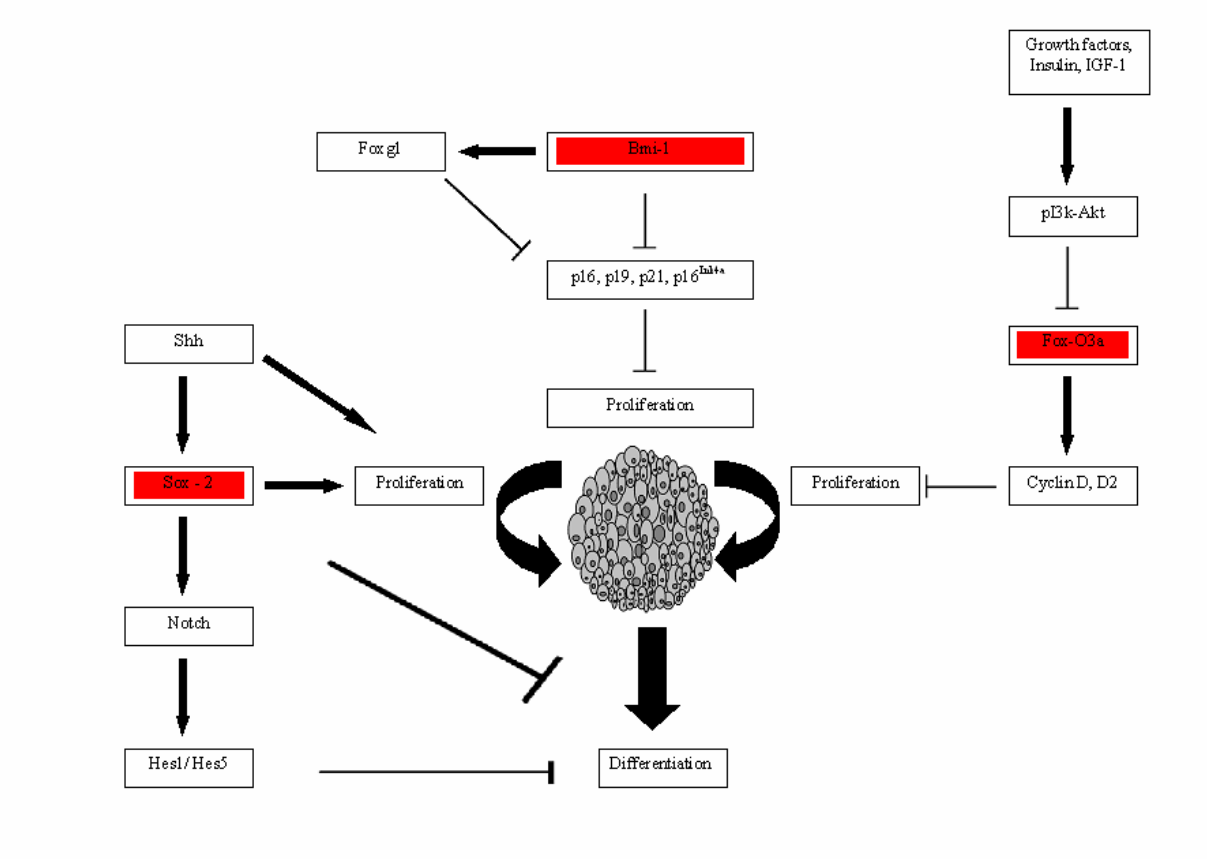


Figure 2. Regulation of neurogenesis.
Neurogenesis is a well orchestrated program and must be regulated precise. The most interesting transcription factors for this thesis are Bmi-1, Sox-2 and Fox-O3a (red). Bmi-1 is necessary for NSC self-renewal and that it represses the cell cycle inhibitors p16, p19 and p21. Bmi-1 further increases the Foxg1 levels specifically in proliferating cells and maintains therefore through the inhibition especially of p21 the NSCs population.
Consecutive expression of Sox-2 maintains neural progenitor characteristics and inhibits differentiation. Through the inhibition of Cyclin D and D2, Fox-O3a reduces proliferation in NSCs. FOX-O3a itself is negatively regulated by the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) signalling pathway, which is activated by growth factors and cytokines, like Insulin and IGF-1.

I.4. NSCs in vitro: Neurospheres biology and the Oligodendrogenic program (OPr)

As it is not possible to study all aspects of the adult neurogenesis *in vivo* isolation of adult NSCs and their following investigation in culture is necessary. Adult neural stem cells can be isolated from several neurogenic and non-neurogenic regions of the adult brain, e.g. SVZ, HC, spinal cord, striatum and neocortex (Reynolds and Weiss, 1992a; Gage et al., 1995; Palmer et al., 1995; Palmer et al., 1999). Usually, the tissue is dissected and dissociated into single cells, which are finally cultured in a defined media supplemented with mitogens, stimulating proliferation like epidermal growth factor (EGF) (Reynolds and Weiss, 1992a) and fibroblast growth factor-2 (FGF-2) (Gritti et al., 1996). Under these proliferation conditions the adult neural stem

cells can be cultured as adherent monolayer cultures or as free floating aggregates, so called neurospheres (NSph) (Reynolds and Weiss, 1992b; Seaberg and van der Kooy, 2002; Wachs et al., 2003b). Through the withdrawal of mitogenic growth factors and / or adding specific factors NSCs can be encouraged to differentiate into mature neural cell types. The resulting differentiation fate can be analyzed by standard immunocytochemical stainings with antibodies directed against antigens specific for one of the three major cell types of the CNS. For the detection of immature neurons the polysialylated- neural-cell-adhesions-molecule (PSA-NCAM) is used, whereas the microtubule associated protein doublecortin (DCX) and microtubule-associated protein 2 isoform a and b (Map2ab) for the location of mature neurons is used (Bernhardt and Matus, 1984; Caccamo et al., 1989; Brown et al., 2003). Astrocytes are detected through the intermediate filament protein GFAP (Delpech et al., 1978; Ghandour et al., 1981) whereas oligodendroglial progenitors can be detected through antibodies against A2B5, platelet-derived growth factor receptorα (PDGFR-α) and NG2 proteoglycan. In addition immature oligodendrocytes can be detected by the expression of galactocerebroside C (GalC) and 2',3'-cyclic nucleotide-3'-phosphohydrolase (CNPase), while mature oligodendrocytes are detected by the expression of the myelin basic protein (MBP). Both, mature and immature oligodendrocytes are immunopositive against the antibody O4 (Knapp et al., 1988; Watanabe et al., 2006). Thus, NSph cultures are useful *in vitro* models to analyze adult neural stem cells properties as well as to explore the properties of NSPCs after the impact of MSC-CM.

As discussed before NSPCs can differentiate in all types of neural cells through the addition of different specific mediators after GFW. Rivera et al. showed that MSC produce soluble factors, which interact with oligodendrogenesis and instruct an oligodendrogenic fate decision on differentiating adult neural stem cells (Rivera et al., 2006).

The production of these oligodendrocytes from NSCs is not a “one step mechanism” but a rather process composed of distinct and hierarchically structured event (Liu et Rao, 2004; Miller, 2002; de Castro et Bribian., 2005). A lot of extracellular matrix components and cell cycle molecules were shown to influence this process. This signals are orchestrated in a program, here termed oligodendrogenic program (OPr).

The paracrine mechanisms of MSCs, which are not restricted to the bone marrow niche also influence the neural stem cell niche. These soluble factors of MSC, pre-

sent in MSC-CM strongly activate and promote the OPr in differentiating rNSCs. Additionally MSC-CM promotes differentiation and maturation of these cells. This was apparently at the expense of astrogenesis, since the number of GFAP-expressing cells was dramatically reduced (Rivera et al., 2010; Rivera et al., 2008; Rivera et al., 2004). To answer the question which factor promotes the effects of MSC-CM on differentiating rNSC several factors have been excluded, like insulin-like growth factor-1 (IGF-1), thyroid hormone (TH), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), transforming growth factor beta-1 (TGF-beta1), neurotrophin-3 (NT-3), sonic-hedgehog (Shh), PDGF-AA, UDP-glucose and Noggin (Rivera et al., 2010; Rivera et al., 2006, Rivera et al., 2008). Importantly, MSCs might act via yet unidentified mechanisms through dictating the intracellular modulators of the OPr (figure 3). In contrast to the observations of Rivera, Bai et al. showed in 2007 that MSCs provide humoral signals that selectively promote the genesis of neurons and oligodendrocytes from mouse derived mouse NSC.

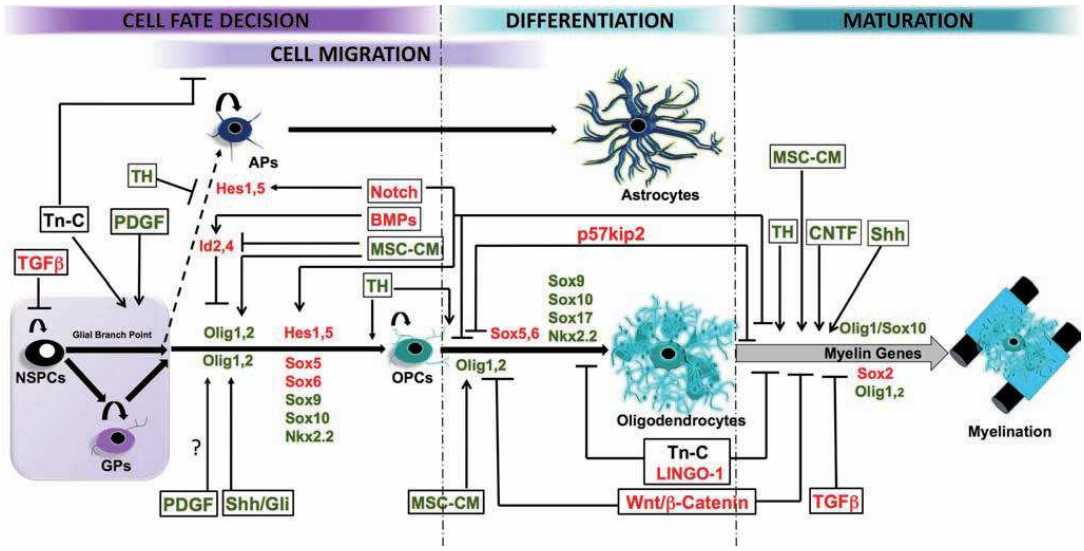


Figure 3. Oligodendrogenesis and the oligodendrogenic program.
Myelinating oligodendrocytes are generated from NSPCs that undergo sequential steps of determination, specification, differentiation, and maturation toward myelinating cells. Proliferating (circular small arrow) NSPCs follow a specification step at the "glial branch point" (denoted in the violet square) and arise as oligodendroglial progenitors (OPCs). This event might occur directly or by an intermediate step in which NSPCs first give rise to proliferating glial progenitors (GPs) that in turn migrate and undergo cell fate decision. Proliferating OPCs migrate and follow a differentiation process toward immature oligodendrocytes. A maturation process, which involves the activation of myelin gene expression, will give rise to myelinating oligodendrocytes. This complete process is highly controlled and regulated by an oligodendrogenic program composed by several factors. These factors function either as oligodendrogenic inhibitors (in red), activators (in green), or both (bifunctional regulators, in black). Extrinsic components (in boxes) stimulate (arrows) or inhibit (T form) intrinsic components (box-free) via signalling pathways. Abbreviations are indicated in the text. (figure extracted from Rivera et al., 2010).

It is one aim of this thesis to characterize the effects of MSC-CM on proliferating NSC and the interaction with the fate decision of this proliferating NSC, in contrast to the existing data, which used differentiating cells for their experiments. Therefore it's known that cells respond different to the same differentiation stimuli, responding on the identity and the intrinsic fate, we investigated if MSC-CM can change this intrinsic fate of NSCs or can prime this cells.

I.5 Reciprocal influence of neural and mesenchymal stem cells

An increasing amount of studies has demonstrated that intravenously infused MSCs are answerable for neuroprotection and regeneration in the lesioned CNS (Dezawa et al., 2001; Hofstetter et al., 2002; Lu et al., 2005; Neuhuber et al., 2005; Zhang et al., 2005; Zhang et al., 2004; Zhang et al., 2004).

For example in the case of MS, several studies have demonstrated that intravenously infused MSCs reduce demyelisation, increase neuroprotection, modulate inflammation and enhance functional recovery (Zhang et al., 2005; Gerdoni et al., 2007; Bai et al., 2009; Barhum et al., 2010; Gordon et al., 2008; Gordon et al., 2010; Kassis et al., 2008; Kemp et al., 2010; Lanza et al., 2009). The underlying mechanisms are still unknown but it might involved one or more of the following possibilities: 1) transdifferentiation of MSCs into functional integrated mature neurons and/or oligodendrocytes (MSCs plasticity); 2) bystander effects of MSCs on the survival of damaged neurons and / or oligodendroglia 3) bystander effects of MSCs on the fate, and differentiation of endogenous NSCs or OPCs present at the lesion site (remyelinistation).

A number of publications have considered the hypothesis that transplanted adult MSCs might transdifferentiate into mature neurons or glial cells, which would integrate into the damaged CNS and promote functional recovery. Although some *in vivo* and *in vitro* studies indicate that MSCs might transdifferentiate into cells from the neural and glial lineage, there is no convincing evidence for the transdifferentiation concept, as described above (chapter I.3.2).

Additionally to the transdifferentiation of MSCs into neuron-like cells, other observations are discussed to be the source of the potential of MSCs, repairing degenerated CNS-tissues. MSCs further promote axonal growth (Neuhuber et al., 2005; Lu et al., 2005; Hofstetter et al., 2002), reduce axonal loss (Zhang et al., 2006) and reduce the

apoptosis of oligodendrocytes (Zhang et al., 2009). In addition to this, the neuroprotective effect of transplanted MSCs is endowed with a strong antioxidant effect *in vivo* (Lanza et al., 2009). In summary, transplanted MSCs enhance neuronal and oligodendroglial survival.

MSCs further enhance the proliferation and differentiation of endogenous neural progenitors *in vivo* and *in vitro* (Zhang et al., 2004). Regarding the underlying mechanisms, Rivera et al., have recently studied the effects of MSCs on NSCs *in vitro* and demonstrated that soluble factors present in conditioned medium from MSCs (MSC-CM) strongly activate and promote the oligodendrogenic process in NSCs, as described above (Chapter I.4) .

Other possible mechanisms for the impact of MSC on NSC include the creation of a favourable environment for regeneration, expression of growth factors or cytokines, vascular effects, remyelinistation or neuroprotection (Parr et al., 2008). It is one purpose of the actual research how these effects of MSCs on NSCs are caused. One possibility that this effects are generated through direct cell-cell interactions between NSCs and MSCs is analyzed through transplantation experiments. After transplantation of MSCs after stroke, contusion or penetrating lesions a remarkable recovery was detected. In a rat stroke model, after MSCs transplantation a significantly smaller volume of damaged tissues was measured and the animals showed a significant functional recovery (Le et al., 2005). Indeed, MSCs and NSCs might be close contact *in vivo*; as a result of the dense network of capillaries present, any position in the brain is within 50µm to the next capillary. Given that MSCs are found in the circulation, the spacing between MSCs and NSCs might be minimal. Moreover, after CNS lesions, the blood-brain barrier becomes leaky and MSCs as well as their secreted factors can penetrate the nervous tissue and act locally (Rivera et al., 2006; Munoz-Elias et al., 2003).

In summary these data show that MSCs appeared as candidate for brain repair that supply large amounts of angiogenetic, antiapoptotic and mitogenic factors to NSPCs, as well as migration towards damaged tissue and neural differentiation of themselves, although the specific underling mechanisms remain controversial and needs to be more explored.

Although the NSPCs are localized in the same regions in the rat and mouse brain, NSPCs seems to be not equal, since they display different properties in response to various substrates, mitogenic growth factors like FGF-2 and EGF, heparin

and to the influence of differentiation factors on the generation of neurons and glia (Ray et al. 2006). It was shown that rNSPCs, cultured in the presence of FGF-2, EGF and heparin lost cluster formation and grew as NSph, whereas mNSPCs attached to the plates, divided and formed monolayer cultures. Only a small number of spheres fails to attach and grows as NSphs. It's established furthermore that that proliferating rNSPCs express mainly glial progenitor markers while mNSPCs more astrocyte/stem cell markers. In addition to this, rNSPCs display an oligodendroglial intrinsic fate while mNSPCs have mainly an astrocyte intrinsic fate. Moreover rNSCs and mNSCs display different electrophysiological properties. mNSPCs showed a resting potential (V_{rest}) that is similar to that of neurons, while rNSPCs had a (V_{rest}) that is similar to that of oligodendrocytes progenitor cells (OPCs) (Steffenhagen et al., 2011). In summary, these data suggest that rNSPCs mainly display an OPC-like identity, whereas mNSPC are composed of a major astrocyte progenitor cell-like population and a minor neuronal progenitor cell-like population.

In consistence with these findings, it seems that MSC have a different impact on rNSPCs as on mNSPCs. Rivera et al. showed that MSC-CM, as far as the co-culture of NSCs and MSCs induces a oligodendrogenic fate decision of differentiating rNSC, which was shown through an increase in the percentage of cells expressing the oligodendrogenic markers GalC and MBP, as through the enhances expression of the oligodendrogenic transcriptional factors Olig1 and Olig2. On the other hand MSC expense an astroglial differentiation of rNSC, whereas no influence on the neural fate decision was detectable (Rivera et al., 2006).

Consistent with the different cell identity, it looks like that MSC-CM displays different effects on adult mNSPCs. Bai et al., analysed if MSCs stimulate the enhancement of migration and the survival of neurospheres-derived cells *in vitro.* It was shown that MSCs promote a neural and oligodendrogenic fate decision in adult mNSPCs, again with an expense of astrocytes. The data showed that under co-culture of MSCs and adult mNSPCs as well as under treatment with MSC-CM a large number of β-tubulin positive neurons and O4 positive immature oligodendrocytes was obtained. However it was demonstrated in the same paper that the effects of MSCs were mediated through soluble factors and were specific for mNSPCs. (Bai et al., 2007).

These differences could be explained through the differences in nature, chemical composition and interactions of a host of intracellular, cell-associated and extracellular matrix proteins or rat and mouse progenitor cells.

II. Aim of the thesis

Adult NSPCs are currently explored for their potential therapeutic use in neuroinflammatory and neurodegenerative brain diseases. They can be obtained from different areas in the adult CNS and be expanded as NSph culture for several passages *in vitro*. These NSph culture are widely used in the filed and are considered as an *in vitro* model for NSPCs and neurogenesis.

Considering the fact that mNSPCs and rNSPCs are no homologous cell population and that MSCs show different impacts on differentiating mNSPCs and rNSPCs this thesis aims to characterize the species-specific effects of MSCs derived soluble factors on proliferating NSPCs of different rodent species - named mouse and rat.

First, the influences of MSCs conditioned media (MSC-CM) on proliferating mNSPCs and rNSPCs were measured through the morphology changes and the bearing of proliferation, cell survival and cell cycle of the NSPCs. Next, the species specific impacts of MSC-CM on proliferating NSPCs were characterized through the phenotype- i.e. the expression pattern of specific markers. Third, the cell intrinsic fate (growth factor withdrawal response) as well as the differentiation potential of NSPC ware examined. The obtained effect was confirmed through the expression of the cell fate determinants.

III. Materials and methods

III.1 Materials

III.1.1. Expendable materials

BD Discardit™ II syringes	Becton Dickinson, Heidelberg
BD Plastikpak™ syringes (1ml)	Becton Dickinson, Heidelberg
Cell culture flasks (50ml, 250ml, 500ml)	TPP, Switzerland
	Greiner Bio-One GmbH, Frickenhausen
Combitips	Eppendorf, Hamburg
Cover glasses Menzel (∅13mm)	VWR, Darmstadt
Cryo Tubes	Nunc, Denmark
Disposable gloves	Hartman, Heidenheim
	Semperit Austria
Microscope slides	Menzel GmbH & Co KG, Braunschweig
Pipette tips	Sarstedt, Nürmbrecht
Pipette tips with filter	Biozym, Hessisch Oldendorf
Syringe-Filter (0.22µm)	TPP, Switzerland
	Millipore, USA
Test plates (24-well, 96-well)	Omnilab, Schubert & Weiß, München
	TPP, Switzerland
Tubes	Eppendorf, Hamburg
	Falcon BD, Heidelberg
	Gibco BRL, Karlsruhe
	Sarstedt, Nümbrecht

III.1.2. Reagents and media for cell culture

a.) Neurobasal (NB) NSph proliferation media

Neurobasal Media	Gibco, Karlsruhe
100 µg/ml Penicillin/Streptomycin	PAN Biotech GmbH, Aidenbach
200 mM L-Glutamin	PAN Biotech GmbH, Aidenbach
1x B27-Supplement	Gibco BRL, Karlsruhe
20 ng/ml Fibroblast Growth Factor (FGF)	R&D Systems, Wiesbaden-Nordenstadt
20 ng/ml Epidermal Growth Factor (EGF)	R&D Systems, Wiesbaden-Nordenstadt
2 µg/ml Heparin	Sigma-Aldrich, Taufkirchen

b.) α-Modified Eagle Media (MEM) MSC – proliferation media

α-MEM	Gibco, Karlsruhe
100 µg/ml Penicillin/Streptomycin	PAN Biotech GmbH, Aidenbach
10% Fetal bovine serum (FBS)	PAN Biotech GmbH, Aidenbach

c.) Knockout- Dulbecco's Modified Eagle Media (DMEM) NSph - differentiation media without serum

Knockout-DMEM Media	Gibco Invitrogen, Karlsruhe
100 µg/ml Penicillin/Streptomycin	PAN Biotech GmbH, Aidenbach
200 mM Glutamin	PAN Biotech GmbH, Aidenbach
20% Serum Replacement (SR)	Gibco, Karlsruhe

III.1.3. Other reagents for cell culture

Accutase PAA,	Pasching, Austria
Dulbecco's PBS	Gibco, Karlsruhe
Laminin	Sigma-Aldrich, Taufkirchen
Poly-L-Ornithin	Sigma-Aldrich, Taufkirchen
Trypanblue	Sigma-Aldrich, Taufkirchen
Trypsin-EDTA solution	Gibco Invitrogen, Karlsruhe

III.1.4. Kits & master mix

Cytotox 96® Non-radioaktive Assay	Promega, Wisconsin USA
FITC-conjugated antibody set (Ki67)	BD Biosciences Pharmigen, San Diego USA
PCR-Kit RNeasy Mini kit	Quiagen, Hilden
Reverse transcription Kit	Promega, Wisconsin USA
TagMan	Applied Biosystems, California, USA

III.1.5. Other chemicals and reagents

Agarose	Invitrogen, Karlsruhe
Bovine Serum Albumine (BSA)	Sigma-Aldrich, Taufkirchen
1-bromo-3-chloropropane (BCP)	Sigma-Aldrich, Taufkirchen
Bromphenol Blue	Sigma-Aldrich, Taufkirchen
DNase I	Quiagen, Hilden
Ethanol	Merck-Schuchard, Hohenbrunn
Ethidiumbromide	Sigma-Aldrich, Taufkirchen

Fish Skin Gelatine	Sigma-Aldrich, Taufkirchen
Glycerol	AppliChem, Darmstadt
HCl	Merck, Darmstadt
Mercaptoethanol	Sigma-Aldrich, Taufkirchen
Paraformaldehyde (PFA)	Sigma-Aldrich, Taufkirchen
PCR marker	NEB, Frankfurt
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Prolong Anti Fade reagent	Invitrogen Molecular Probes™ Eugene, Oregon, USA
Propidium-iodite	Sigma-Aldrich, Taufkirchen
RNase A	Roche Diagnostics
Sodium hydrogen carbonate	Merck, Darmstadt
Tris-Base	Sigma-Aldrich, Taufkirchen
Triton 100-X	Sigma-Aldrich, Taufkirchen

III.1.6. Buffer, solutions and stock solutions

a.) Fish Skin Gelatin Buffer (FSGB)	0.1M Tris-HCl, pH 7.5
	0.15M NaCl
	1% w/v BSA
	0.2% v/v Fish Skin Gelatin
	Optional: 0.1% v/v Triton X-100

b.) 4% Paraformaldehyde (PFA)	4% PFA
	2.5mM NaOH
	0.4mM CaCl2
	50mM Sucrose
	0.1M NaH2PO4

III.1.7. Devices

Device	**Company**
Centrifuge 5417 R	Eppendorf, Hamburg
E-Max precision microplate reader	Molecular Device Corporation, USA
FACSCalibur	Becton Dickinson
Fluorescence Microscope Leica DMR with SPOT Camera	Leica, Solms Diagnostic Instruments, USA
Incubator	HERA Cell
Inverse Fluorescence microscope	Olympus, Hamburg
Light microscope Olympus CK 30	Olympus, Hamburg
Megafuge 1.0 R	Heraeus Instruments GmbH, Germany
Olympus IX 70 with Color View	Soft Imaging Systems, Münster
	documentation system
Photometer Ultrospec 2000	Amersham/Pharmacia Biotech, Freiburg
Rotor-Gene 6000 R Corbett Research	geneXpress, Vienna, Austria

III.1.8. Software

Adobe PhotoshopC2 Version 9.0	Adobe Systems GmbH, München
EndNote 7	Thompson ResearchSoft, USA

GraphPad Prism	GraphPad Software Inc., USA
Microsoft Office	Microsoft Corporation
Softmax. Version 2.34	Molecular Device Corporation, USA
WinMDI2.8	Microsoft Corporation

III.2. Cell culture methods

III.2.1. MSC cultures

Six to eight week old female Fisher rats were killed by cervical dislocation. Femurs and tibias were obtained from the legs after the muscular tissue was removed. Bone marrow plugs harvested from femur and tibia were resuspended in approximately 10ml of α-MEM, desegregated, homogenized and recovered by centrifugation (800 x g). The resulting cell pellet was resuspended in α-MEM containing 10% FBS (α-MEM- 10%FBS). The cell number was determined by Trypan blue exclusion and the cells were seeded at $1x10^6$ cells/cm^2 into 56 cm^2 culture dishes in the following. Cells were incubated in a humidified incubator at 37℃ wi th 5% CO_2. After three days, non-adherent cells were washed off and adherent cells were further incubated in fresh α-MEM-10%FBS until a confluent layer of cells was reached. Afterwards, cells were trypsinized using a 0,25% Trypsin-EDTA solution, resuspended in α-MEM-10%FBS and seeded at a density of 8000 cells/cm_2. After three to five days, the resulting monolayer of cells was again trypsinized and frozen or further cultured for experiments (Santa Maria et al., 2004).

III.2.2. Preparation of NSCs primary cultures

Six to eight week old female Fisher rats and C57BL/6 (Charles River Deutschland GmbH, Germany) mice were anesthetized with ketamine and decapitated. Brains were removed and collected in ice cold Dulbecco's PBS (DPBS). HC and SVZ were taken out. To remove the PBS brain pieces were centrifuged for 5 minutes at 120 x g

at 4℃. Then, the dissected tissue was mechanically dissociated with a razor blade. In order to wash the tissue with PBS it was transferred back into the tube, PBS was added and it was centrifuged again for 5 min at 120 x g and 4℃. The pellet was re-suspended in 10 ml PPD-solution containing 0,01% Papain, 0,1% dispase II, 0,01% DNase I and 12,4 mM $MgSO_4$ in HBSS without Mg^{2+}/Ca^{2+}. Cells were digested for 30 to 40 minutes in the PPDsolution, triturating every 10 min. After further centrifugation like mentioned before, supernatant was removed. The pellet was resuspended in serum-free Neurobasal media (NB) containing B27-supplement, 2 mM L-glutamine and 100 U/ml penicillin/ 0,1 mg/l streptomycin and cells were centrifuged again. This step was repeated once more. At the end the pellet was resuspended in NB media supplemented with B27-supplement, 2 mM L-glutamine, 100 U/ml penicillin/ 0,1 mg/l streptomycin, 2 µg/ml heparin, 20 ng/ml EGF and 20 ng/ml FGF. Cells were plated into T-25 culture flasks and maintained at 37℃ in a humidified incubator with 5% CO_2.

III.2.3. Cultivation and passaging of adult NSCs

NSCs were cultured in T-75 cell culture flasks with a density of 5 x 10^4 cells/ml in NB media. Media were changed 3-4 days after seeding. To passage the NSph, the culture media containing the floating NSph was collected in a 15 ml tube and centrifuged at 120 x g for 5 min. For dissociation, the pellet was resuspended in 200 µl Accutase and triturated using a pipette tip. The cell suspension was incubated at 37℃ for 10 min in the water-bath. To stop the Accutase reaction 0.8 ml media were added and the cells were dissociated mechanically by aspirating and expelling the suspension with a pipette. Cell number was determined by Trypan blue exclusion and 5 x 10^4 cells/ml were plated in new T-75 culture flasks in NB media. NSPCs used in this study were derived from cultures of passage number four to eight.

III.2.4. Cell counting

For estimating proliferation of NSphs by assessing cell number, 5 x 10^4 cells were seeded in T75 cell culture flasks either with Neurobasal medium, or with a mixture

50% Neurobasal medium and 50% MSC-CM (50% MSC-CM), or only with MSC-CM (100% MSC-CM). The cells were cultivated in these mediums and were determined by Trypan blue exclusion after 7, 14 and 21 days. Media were changed after three to four days.

III.2.5. Preparation of MSC-conditioned media

Mesenchymal stem cell conditioned medium (MSC-CM) was prepared similar as described in Rivera et al., 2006 with the exception of the media used. MSCs were plated at 12,000 cells/cm^2 and incubated in normal NSCs proliferation medium (NB medium supplemented with B27 (NB/B27), 2 mM L-glutamine, 100U/mL penicillin/ 100 µg/ml streptomycin, 2 µg/ml heparin, human recombinant 20 ng/ml bFGF-2 and human recombinant EGF 20 ng/ml). After 3 days, the conditioned medium was collected and filtered using a 0.22 µm-pore filter. In some of the experiments, the conditioned medium was replenished with EGF and b-FGF (20 ng/ml each) to exclude the possibility that MSCs might have consumed the growth factors.

III.2.6. Coating of coverslips with poly-L-ornithin and laminin

Glass-coverslips (13 mm) (Menzel GmbH, Braunschweig, Germany) were incubated in 1 M HCl at 65℃ overnight and stored in isopropanol until further use. Coverslips were put into 24-well test plates and air-dried. Coverslips were incubated for at least 2 h with 100 µg/ml Poly- L-ornithin solution at 37℃ in the following. After washing three times with sterile water coverslips were incubated with 5 µg/ml laminin solution for 2 h. Immediately after removal of the laminin solution cells were seeded.

III.2.7. Phenotype and fate analysis of NSph

To assess the potential of various factors to change phenotype and fate of adult rNSph and mNSph cells were incubated for three weeks under proliferation conditions (NB-Medium [Control], 50% MSC-CM and 100% MSC-CM. Media were

changed every three to four days and after seven days the NSph cultures were passaged like mentioned before (s. II.2.3.) After three weeks of incubation, 2-5 x 10^4 cells/well were plated on poly-L-ornithine (100 µg/ml) and laminin (5 µg/ml)- coated glass coverslips (s. II.2.6.) into 24-well plates in DMEM Knockout-20% SR. After 12 h, cells were either directly fixed for 30 min with 4% Paraformaldehyde and processed for immunofluorescence staining or media was refreshed for further incubation of the cells. After 7 days, the remaining cells were fixed for 30 min with 4% Paraformaldehyde and processed for immunofluorescence staining.

III.2.8. Immunofluorescence analysis and quantification

First, the fixed cells were washed three times in Phosphate-buffered saline (PBS) and then blocked with Fish Skin Gelatin Buffer (FSGB) to occupy unspecific binding sites for 2 h at room temperature. If staining should be performed for a cyctoplasmic marker the use of FSGB containing 0.1% Triton X-100 was necessary in order to make the cell membrane permeable for the antibody. The same blocking solution was also used for the dilutions of the antibodies. The primary antibodies were applied overnight at 4℃.

The following antibodies and dilutions were used:

a.) Primary Antibodies

Primary Antibodies	**Dilution**	**Company**
rabbit anti-NG2	1:200	Millipore, USA
IgM mouse anti-O4	1:100	Chemicon, USA
IgM mouse anti-A2B5	1:100	Chemicon, USA
rabbit anti-platelet-derived growth factor receptor (PDGFRα)	1:100	Santa Cruz, Heidelberg
IgM mouse anti-polysialylated-neural-cell-adhesions-molecule (PSA-NCAM)	1:500	Millipore, USA

rabbit anti-glial fibrillary acidic protein (GFAP)	1:1000	Dako, Glostrup, Denmark
mouse anti-myelin basic protein (MBP) (SMI94)	1:750	Hiss- Freiburg
rabbit anti-doublecortin (DCX)	1:500	NEB, Frankfurt
mouse anti-rat nestin	1:500	Pharmingen, Heidelberg
mouse anti 2', 3'-cyclic-nucleotide-3'-phosphodieesterase (CNPase)	1:200	Millipore, USA
mouse anti-microtuble-associated-protein 2a+b (Map2a+b)	1:400	Sigma-Aldrich, Taufkirchen

Table 1 Primary antibodies used

After washing three times with FSGB, cells were incubated with the species-specific secondary antibodies, which were conjugated to fluorochromes, for 2 h in the dark at room temperature. The following antibodies and dilutions were used:

b.) Secondary Antibodies

Secondary Antibodies	**Dilution**	**Company**
donkey anti-mouse, -rabbit conjugated with rhodamine X (RHOX); IgG and Ig M	1: 500	Dianova, Hamburg
donkey anti-mouse, -rabbit conjugated with Alexa Fluor®□488	1:1000	Invitrogen Molecular Probes, Eugene, Oregon USA

Table 2 Secondary antibodies used

To remove unbound secondary antibodies, cells were washed again with PBS. The cell nuclei were counterstained with 4', 6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 0,25 µg/µl diluted in PBS. Finally, cells were washed again three times with PBS and were mounted on microscope slides using Prolong Antifade reagent.

For the simultaneous detection of two different antigens the used primary and secondary antibodies were incubated simultaneously. For analysis and quantification, epif-

luorescence was observed via a Leica microscope and documented by a coupled Spot digital camera. Three to seven observation fields were selected randomly and photographed for cell fate analysis.

III.2.9 FACS analysis (Ki67)

mNSCs were incubated with NB-Medium (Control), 50% MSC-CM and 100% MSC-CM under normal proliferative conditions for 7, 14 and 21 days. After the accordant days the cells were dissociated into single cells with Accutase. After 10 min incubation at 37℃, Accutase was exhausted and cells were resuspended in 1 ml of ice-cold 70% ethanol. They were kept overnight at -20℃. At the earliest one day later, cells were processed for staining according to a protocol of Endl (Endl et al., 1997) which was slightly modified. At first, 2 ml of ice-cold PBS were added to each sample. Cells were centrifuged (600 x g for 5 min), resuspended in 500 µl ice-cold PBS containing 0.1% Triton X-100 and incubated for 5 min on ice. Cells were washed twice by adding 2 ml of ice-cold PBS followed by centrifugation for 5 min at 600 x g. Pellets were then resuspended in 120 µl PBS containing either FITC conjugated mouse anti-human Ki67 or mouse IgG control antibody (100 µl PBS+20 µl antibody solution) and incubated at room temperature for 30 to 45 min in the dark. Afterwards samples were washed by adding 1 ml of PBS. They were centrifuged, supernatant was aspirated and cells were resuspended in 470 µl of PBS and 5 µl of RNase A (stock 1 mg/ml). After a 60-minute incubation at 37℃, 25 µL of propidium-iodide (stock 1 mg/ml) were added and samples were analyzed on a FACSCalibur flow cytometer. Data were processed using WinMDI 2.8 software.

III.2.10 CytoTox96®Non-Radioactive Cytotoxicity Assay

mNSCs were incubated with NB-Medium (Control), 50% MSC-CM and 100% MSC-CM under proliferative conditions for 7, 14 and 21 days. After the accordant days the cells were centrifuged at 240 x g for 5 min. Aliquots of 50 µl from all tubes were carried over a fresh 96-well plate and 50 µl substrate mix were added to each well of the plate. Cells were incubated at room temperature for 30 min under protection from

light. Subsequently 50 µl of stop solution were added to each well before the absorbance was recorded at 490 nm.

III.3 Molecular Methods

III.3.1. RNA – Extraction from Neurospheres

RNA extraction from adult NSph was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany). Cells were spun down and the pellet was lysed in RLT buffer supplemented with 0,1 M 2-Mercaptoethanol (1:100 dilution). The lysate was homogenized by aspirating and expelling the suspension with a pipette vigorously. The homogenate was mixed with 350 µl 70% Ethanol and was loaded onto the RNeasy spin column by centrifugation at 10000 x g for 1 min. After washing the sample with 350 µl RW1 buffer and centrifugation at 10000 x g for 1 min, DNA digestion was performed in order to remove genomic DNA contamination. For that, a mixture of 10 µl DNase I and 70 µl RDD buffer (Qiagen, Hilden) was added to each column and incubated for 15 min at room temperature. After the incubation, columns were washed again with 350 µl RW1 buffer to remove the DNase.

Afterwards, columns were washed with RW1- and RPE buffer followed by centrifugation at 10000 x g for 1 min to get rid of contaminations of the RNA. To dry the silica gel membrane columns were placed into new 2 ml collection tubes and centrifuged at 10000 x g for 2 min. Finally, RNA was eluted by adding 30 µl of RNase free water to the columns and centrifuging at 10000 x g for 1 min. To get a higher concentration of RNA the elution step was repeated using the eluate from the step before.

For determination of RNA concentration, the RNA was diluted with H_2O (1:25) and the absorbance was measured with a photometer. RNA was stored at -80°C until cDNA synthesis was performed.

III.3.2 cDNA synthesis

cDNA synthesis was performed using Promega reverse transcription Kit. 1 µg of RNA was used for the reverse transcription into cDNA. The sample was filled up to a volume of 9 µl with RNase free H2O. The mixture was put into the thermocycler (Eppendorf, Hamburg) for 10 minutes at 70℃. While on 4℃ for 10 minutes 11 µl of the Master Mix were added. The

Master Mix contained:

4 µl MgCl2 (4 mM)
2 µl dNTP (800 µM)
2 µl Reverse Transcription 10 x buffer (100 mM Tris-HCl, (pH 8.8); 500 mM KCl,
1% Triton X-100)
1 µl Random Primers (20 ng/µl)
0.7 µl AMV Reverse Transcriptase (5 U/ml)
0.5 µl RNase Inhibitor (40 U/ml)
0.8 µl H2O
Σ 11 µl

After adding the master mix the temperature profile was the following:

10 min 21℃ 1x
15 min 42℃ 1x
05 min 95℃ 1x
05 min 4℃ 1x

The final cDNA equivalent concentration was 50 ng/µl (1 µg/20 µl). cDNA was stored at -20℃.

III.3.2 Quantitative PCR

Expression analysis was performed by TaqMan gene expression assays kits (Applied Biosystems, California, USA) for the following rat genes: olig2 and Id2. Probes and primers were provided by the manufacturer (Applied Biosystems, California, USA). Rat β2-microglobulin was used as endogenous normalized gene. The following temperature profile was used: activation of polymerase 95 C, 10 min; 40 cycles of denaturing 95 C, 15 s, and annealing/extension 60 C, 60 s. Data was obtained with a Rotor-Gene 6000 R Corbett Research (geneXpress, Vienna, Austria) and analyzed by delta delta Ct method (Livak and Schmittgen, 2001). Gene expression under control conditions was used as a calibrator gene. Finally, the expression value of Olig2 and Id2 was determined for each condition. To determine the Olig2/Id2 ratio, Id2 was used as a calibrator gene for each condition.

III.3.3 Gel electrophoresis of DNA

Separation of DNA fragments was performed via Agarose gel electrophoresis (1,5 % Agarose in TBE). Additionally to the DNA a DNA ladder was applied to determine the size of the DNA fragments after the electrophoresis. Electrophoresis occurred at the constant voltage of 96V. The Agarose gel was supplemented with an Ethidiumbromide solution which intercalated into the DNA so that the DNA fragments could be detected under UV-light.

III.4. Statistics

Data are presented as means ± SD and statistical analysis was performed using PRISM4 (GraphPad, San Diego, CA, USA). P values of $< 0,05$ were considered to be significant acquired by parametric one-way ANOVA-Tukey post hoc. All experiments were performed in triplicate or more.

IV. Results

MSCs have been demonstrated to promote functional recovery in a number of neurological diseases in animal models (f.e. Lee et al. 2008). In the respect it was shown that soluble factors derived from MSCs induce an oligodendrocyte fate on adult NSCs under serum containing conditions (Rivera et al., 2006). Here, we test the hypothesis that MSCs produce soluble factors that might influence the biology of neural stem/progenitor cells undergoing proliferation. To reach this reason we performed all experiments in serum free conditions, to get the cells in proliferation.

The effects of MSC-CM on proliferating adult rNSph and mNSph were analyzed regarding cell morphology, proliferation, survival, cell cycle, cell phenotype (cell specific marker and cell fate determinant expression profile) and growth factor withdrawal response.

IV.1. Effects of MSC-CM on proliferating rNSphs

IV.1.1. MSC-CM promotes adhesion of proliferating rNSphs and affects cell morphology

MSC-CM was used to study the effect of soluble factors derived from MSCs on proliferating rNSphs. rNSphs were incubated under normal proliferating conditions (control), 50% MSC-CM or 100% MSC-CM. After 7, 14 or 21 days cell morphology was analyzed at the Olympus microscope. Proliferating rNSphs grew as floating aggregates, when they were cultured in normal proliferating medium (control) (figure1 A, D, G). However, when rNSphs were incubated with 50%MSC CM or 100% MSC-CM, they started to attach to the plate and primary and secondary process were observed independently from incubation time (figure 4 B, C, E, F, H, I).

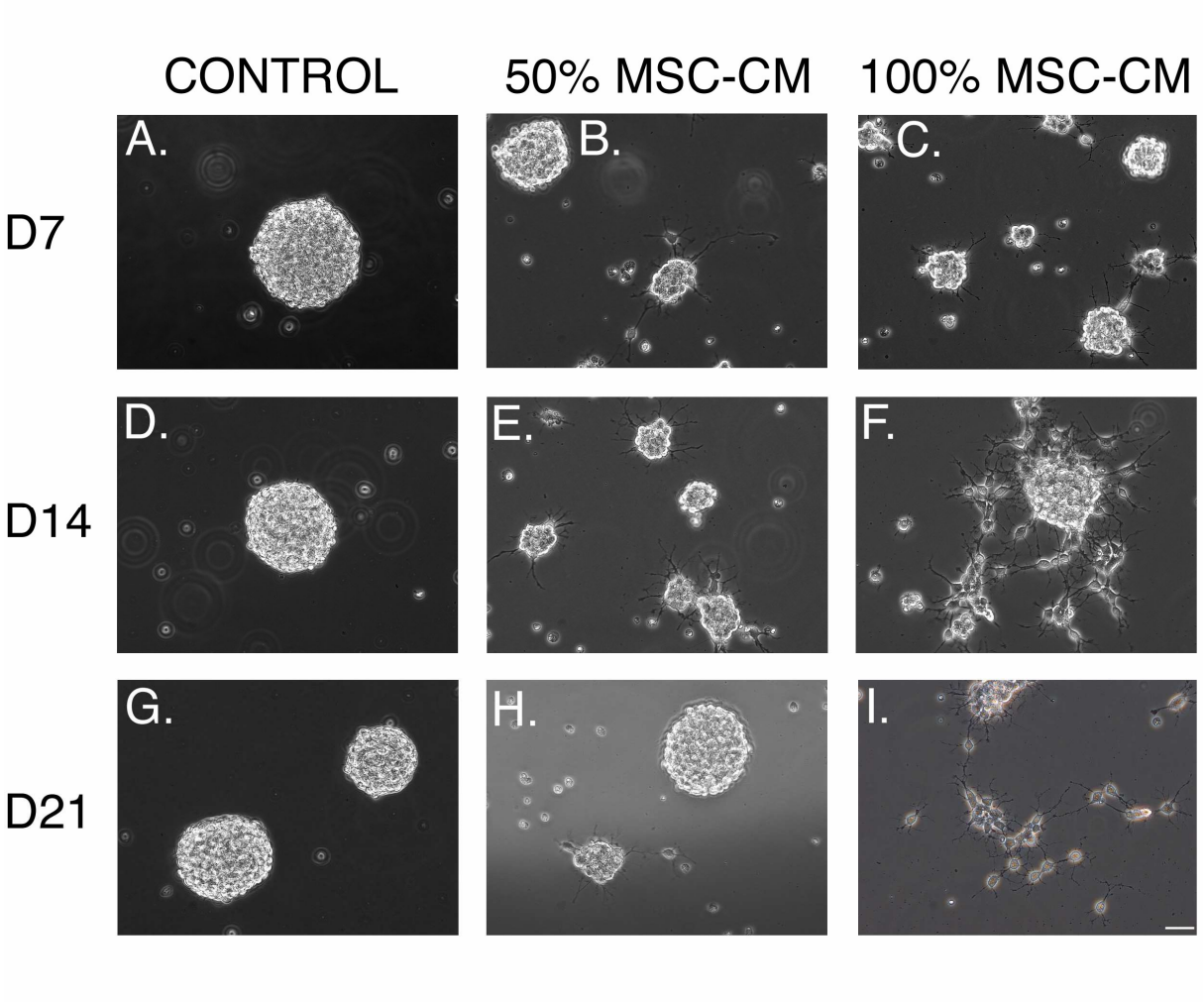


Figure 4. Morphology of proliferating rNSphs.
Phase contrast images of rNSphs in normal proliferation medium (control) **(A,D,G)**; treated with 50% MSC-CM medium **(B,E,H)** or with 100% MSC-CM **(C,F,I).** Proliferating rNSphs were incubated during 7 days **(A-C)**, 14 days **(D-F)** and 21 days **(G-I)**. Note that under control conditions proliferating rNSphs grew as floating aggregates, however when the cells were treated with 50% or 100% MSC-CM some rNSphs attached to the surface of the flask and grew adherent . Scale bar = 100 µm.

IV.1.2. MSC-CM has no effect on the absolute number of rNSphs in vitro

To detect any influence of MSC-CM on the growth characteristics of proliferating rNSphs, cells were incubated under normal proliferating conditions (control), 50% MSC-CM or 100% MSC-CM for 21days with a change of media every 3-4 days. After 7, 14 and 21 days cell number was analyzed by Trypanblue exclusion.

The cells grew exponentially after seeding, equal which media were used (control, 50% MSC-CM or 100% MSC-CM). It was not possible to detect any significant difference in the absolute number of cells nor any significant difference in the kinetics between the three media (figure 5 A, B, C).

These data indicated that MSC-CM has no detectable influence on the proliferation of proliferating rNSphs.

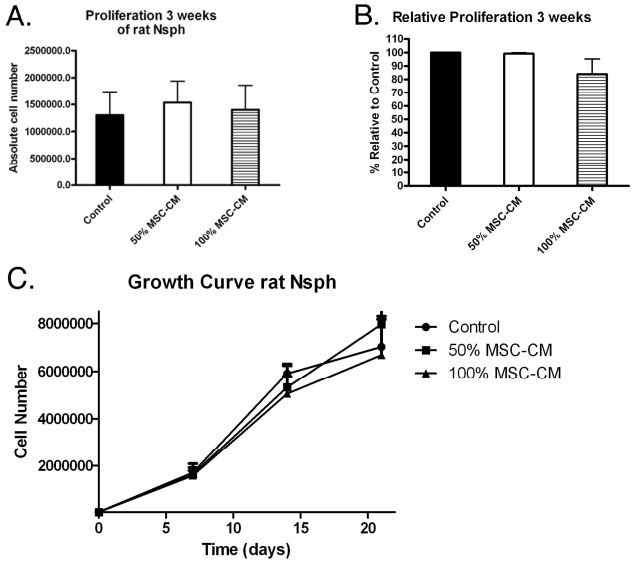


Figure 5. MSC-CM has not any significant influence on the growth-bearing of proliferating rNSphs.
rNSphs were incubated in normal proliferation medium (control), 50% MSC-CM and 100% MSC-CM, to check the influence of MSC-CM on the proliferation of proliferating rNSphs. Cell number was analyzed by Trypanblue exclusion after 7, 14 and 21 days. It was not possible to detect any significant difference in the growth-bearing and the proliferation rate of proliferating rNSphs. These data indicated that MSC-CM has no detectable influence on the proliferation of proliferating rNSphs.

IV.1.3. MSC-CM does not affect the highly expression pattern of glial and oligodendroglial progenitor markers on proliferating rNSphs

To determine whether MSC-CM affects the expression pattern of cell lineage specific markers, non-treated (control) and MSC-CM-treated proliferating rNSphs were dissociated. After dissociation single cells were seeded overnight under serum-free conditions to allow them to attach. After this procedure cells were fixed and marker expression was analyzed in the following.

Most of the cells derived from proliferating control rNSphs expressed the oligodendroglial progenitor marker O4 (approximately 90% figure 6 G, H), the glial progenitor markers A2B5 (approximately 50% figure 6 C, D) and NG2 (approximately 30% figure 6 E, F). MSC-CM-treated proliferating rNSphs displayed no difference on the percentage of cells that express glial and oligodendroglial progenitor markers compared to non-treated proliferating rNSphs (figure 6 D, F, H). In addition to this, a small sub-

population of cells derived from proliferating rNSphs expressed astrocyte/neural stem cell markers GFAP and Nestin (between 5 to 15% figure 6 A, B). MSC-CM treatment seemed to not affect the expression of the astrocyte/neural stem cell phenotype with the exception on the percentage of GFAP-expressing cells. There a slightly increase was detected (Fig 6 B). Finally, there were few cells that express the neuronal progenitor marker DCX on normal proliferating rNSphs (less than 2% figure 6 I, J). MSC-CM treatment did not affect the DCX expression (figure 6 J).

In summary these findings suggested that soluble factors derived from MSCs did not affect the major expression pattern of glial and oligodendroglial markers on proliferating rNSphs.

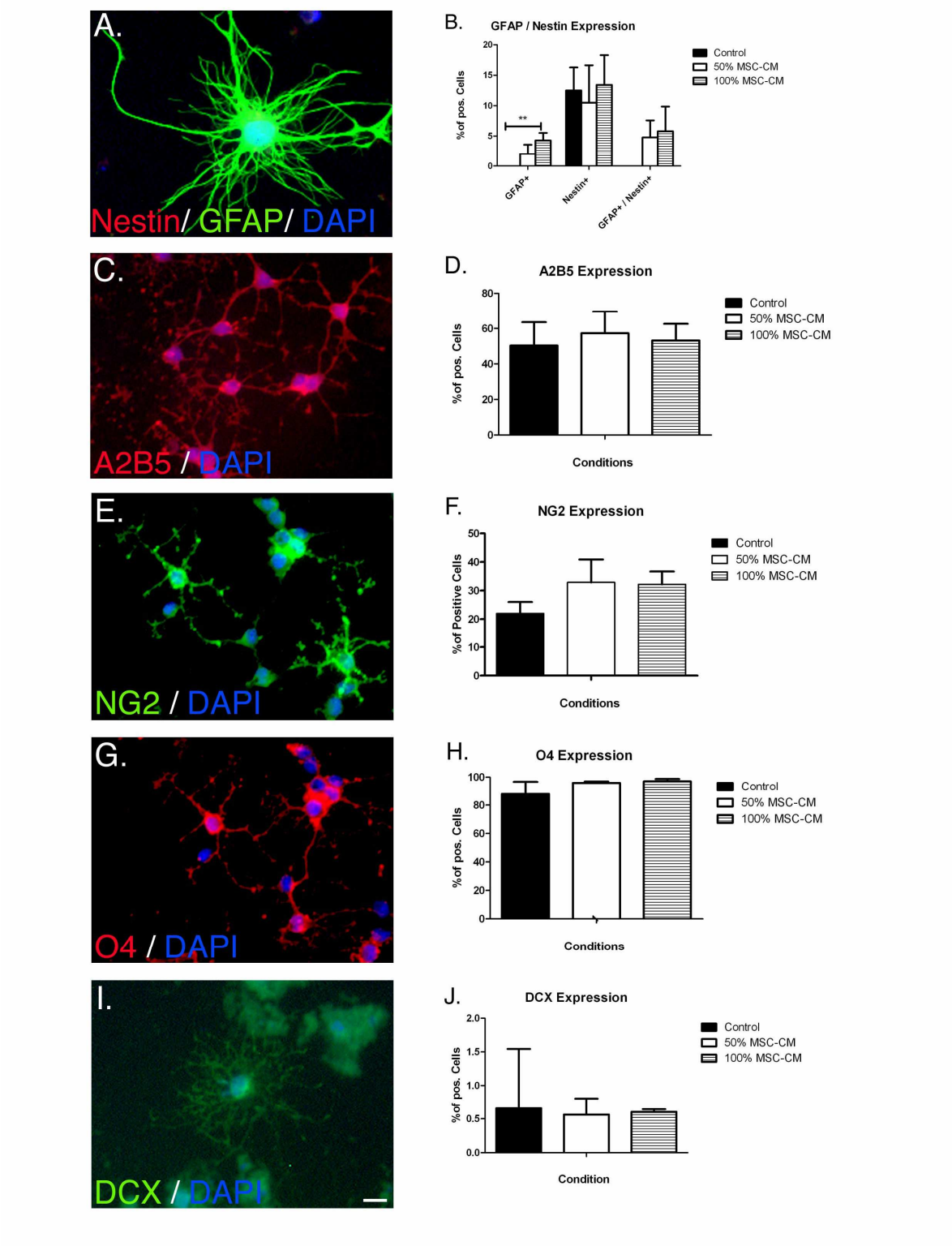
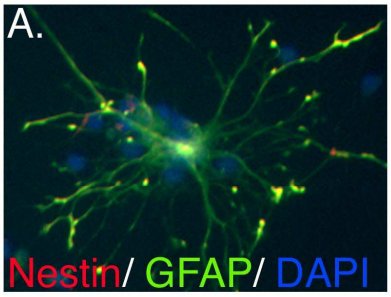


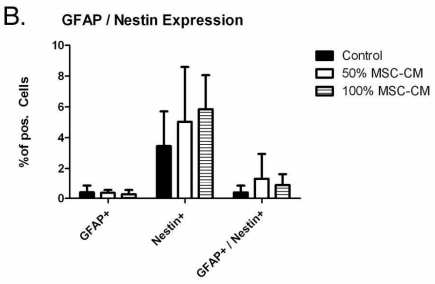
Figure 6. Marker expression profile of proliferating rNSphs pre-incubated 21 days under control condition, 50% MSC-CM and 100% MSC-CM.
Treated rNSphs were dissociated and cells were seeded overnight under serum-free conditions. One day after the cells were fixed and stained for immunohistochemistry. The marker expression was analyzed by immunofluorescence. Illustrative fluorescence images are shown for Nestin (red), GFAP (green) and DAPI (blue) **(A)**; A2B5 (red) and DAPI (blue) **(C)**; NG2 (green) and DAPI (blue) **(E)**; O4 (red) and DAPI **(G)**; and DCX (green) and DAPI (blue) **(I)**. Quantitative analysis for each marker are shown (B, D, F, H and J). Note that there was no significant change between the different conditions for any marker tested with the exception of GFAP. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. Scale bar = 100µm

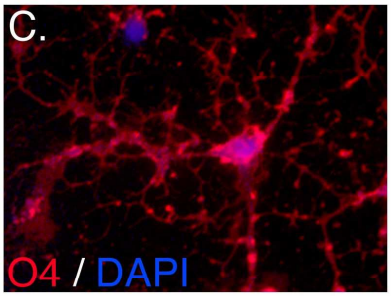
IV.1.4. MSC-CM enhances rNSphs oligodendrogenic response after growth factor withdrawal

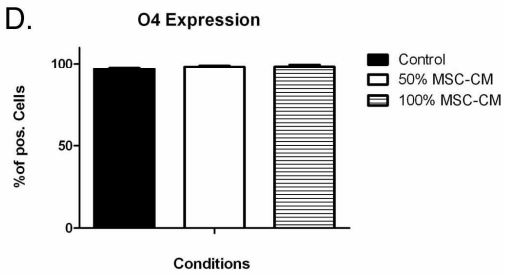
In a previous study it was illustrated that rNSphs spontaneously differentiate into oligodendrocytes after GFW (Steffenhagen et al., 2011). To determine whether MSC-CM affects this oligodendrogenic response after GFW, non-treated and MSC-CM-treated proliferating rNSphs were dissociated and seeded overnight under serum-free conditions and incubated for 7 days without growth factors (FGF-2 and EGF).
As expected, one week after GFW, dissociated non-treated rNSphs spontaneously differentiate into mature oligodendrocytes since approximately 60% of the cells of the control group expressed the oligodendrocyte mature marker MBP (figure 7 G, H). Interestingly, although MSC-CM treatment on proliferating rNSphs did not affect the proportion of O4-expressing oligodendrocyte progenitor cells (figure 7 C, D) it increased the percentage of CNPase- and MBP-expressing mature oligodendrocytes in response to GFW (figure 7 E, F and G, H respectively).
It was not possible to observe a difference on the percentage of cells that display astrocyte/neural stem cell phenotype on MSC-CM-treated rNSphs compare to control, since Nestin and GFAP expression was not affected after GFW (figure 7 A, B). Finally, there were few cells that expressed the neuronal progenitor marker DCX in response to GFW (less than 4% figure 7 I, J) and MSC-CM treatment on proliferating rNSphs decreased the percentage of DCX expressing cells (figure 7 J) but did not affect the proportion of Map2ab-positive mature neurons generated after GFW (less then 1% figure 7 K, L).

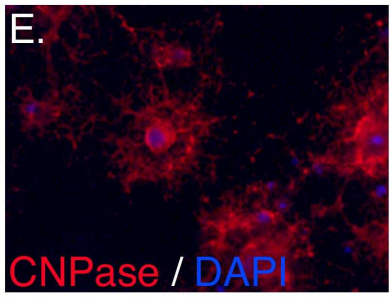
In summary, these data support that MSC-CM enhances the oligodendrogenic ability of proliferating rNSphs in response to GFW.

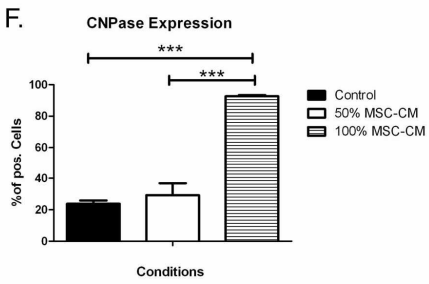

A.
Nestin/ GFAP/ DAPI

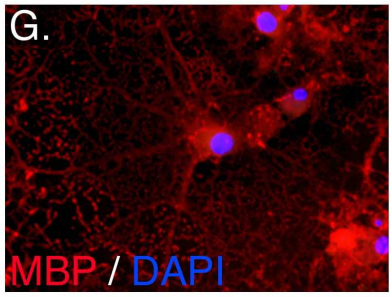

B.
GFAP / Nestin Expression
%of pos. Cells
10
8
6
4
2
0
GFAP+
Nestin+
GFAP+ / Nestin+
Control
50% MSC-CM
100% MSC-CM

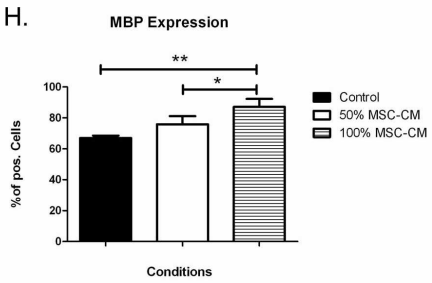

C.
O4 / DAPI

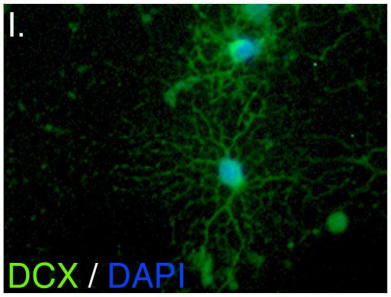

D.
O4 Expression
%of pos. Cells
100
50
0
Conditions
Control
50% MSC-CM
100% MSC-CM

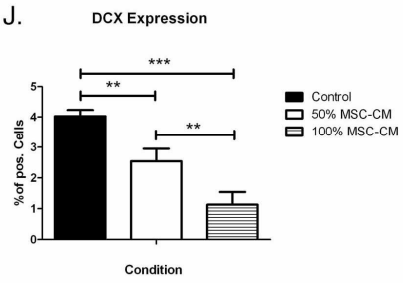

E.
CNPase / DAPI


F.
CNPase Expression
***
***
%of pos. Cells
100
80
60
40
20
0
Conditions
Control
50% MSC-CM
100% MSC-CM


G.
MBP / DAPI


H.
MBP Expression
**
*
%of pos. Cells
100
80
60
40
20
0
Conditions
Control
50% MSC-CM
100% MSC-CM


I.
DCX / DAPI


J.
DCX Expression
***
**
**
%of pos. Cells
5
4
3
2
1
0
Condition
Control
50% MSC-CM
100% MSC-CM

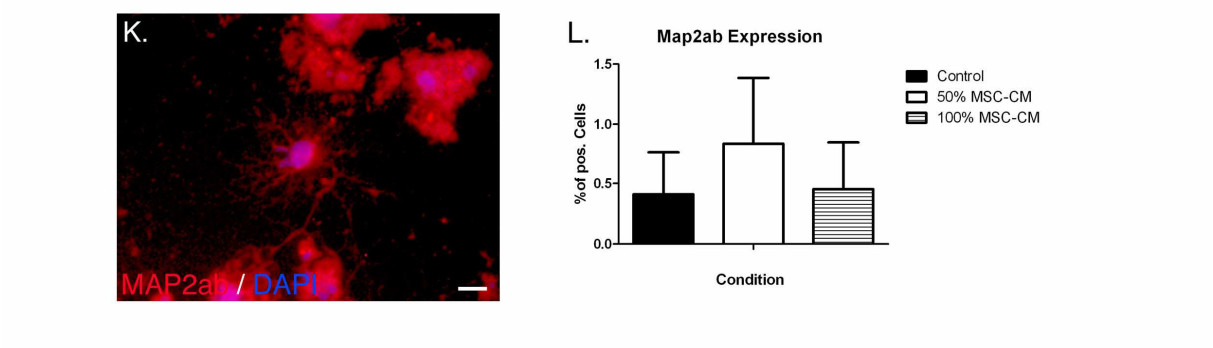


Figure 7. Growth factor withdrawal response of rNSphs pre-incubated 21 days under control condition, 50% MSC-CM and 100% MSC-CM.
Treated rNSphs were dissociated, seeded and incubated for one week under serum-free conditions without growth factors. After 21 days cells were fixed and stained for immunohistochemistry. Marker expression was analyzed by immunofluorescence. Illustrative fluorescence images are shown for Nestin (red), GFAP (green) and DAPI (blue) **(A)**; O4 (red) and DAPI (blue) **(C)**; CNPase (red) and DAPI (blue) **(E)**; MBP (red) and DAPI **(G)**; DCX (green) and DAPI (blue) **(I)**; and MAP2ab (red) and DAPI (blue) **(K)**. Quantitative analyses for each marker are shown in the left panels (B, D, F, H, J and L). Note the significant increase in the percentage of cells that express the oligodendroglial markers CNPase and MBP when cells were pre-incubated with 50% and 100% MSC-CM compared with the control condition. There is also a significant decrease in the percentage of cells that express the neuronal marker DCX in 50%MSC-CM and 100% MSC-CM compared with the control medium. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. Scale bar = 100µm

IV.1.5. MSC-CM primed the oligodendrogenic program on proliferating rNSphs

First in order to verify if proliferating rNSphs response to GFW activating an oligodendrocyte differentiation program, the expression pattern of oligodendrogenic markers on proliferating rNSphs and one week after GFW has been compared.

The expression pattern of the glial progenitor markers A2B5 (figure 8 A, from approximately 60% to less than 5%) and PDGFα (figure 8 B, from approximately 30% to less than 5%) were drastically reduced, while the oligodendrocyte mature marker MBP was significant increased in response to GFW (figure 8 F, from less than 5% to approximately 60%). In addition, the high expression of the oligodendrocyte progenitor and immature marker O4 was maintained after GFW (figure 8 D). These observations suggest an activation of an oligodendrocyte differentiation program in response to GFW, in which the expression of early oligodendrogenic markers switch-off while late markers switch-on. MSC-CM-treated proliferating rNSphs displayed a higher capacity to switch-off the glial progenitor marker NG2 after GFW compared to non-treated proliferating rNSphs (figure 8 C). Moreover, as was previously mentioned MSC-CM treatment increased the percentage of CNPase- and MBP-expressing cells in response to GFW compared to control conditions (figure 8 E, F). In addition to this,

MSC-CM treatment seemed to increase also the percentage of MBP-expressing cells under proliferative conditions respect to control (figure 8 F).

In conclusion, these data suggest that soluble factors derived from MSCs predispose or prime the oligodendrogenic program of proliferating rNSphs and therefore enhancing oligodendrogenesis.

In a further trial of experiments, it was tested if oligodendroglial priming of proliferating rNSphs by MSC-CM could induce an altered expression of pro- and anti-oligodendrogenic fate determinants. Here, the relative expression of Olig2 and Id2 is a key determinant for oligodendrocyte / astrocyte fate decision. Therefore, proliferating rNSphs were incubated up to 3 weeks under normal proliferating conditions (control), 50% and 100% MSC-CM, the expression levels of Olig2 and Id2 were analyzed by quantitative RT-PCR and the Olig2/Id2 ration was determined for the different conditions. Although the expression levels of Olig2 did not change within the different conditions (figure 8 A), 50% and 100% MSC-CM drastically reduced the expression of Id2 (figure 8 B). In consistence, MSC-CM increased the Olig2/Id2 ratio compared to control conditions (figure 8 C) favouring oligodendrocyte fate decision. Moreover, the increased Olig2/Id2 ratio in MSC-CM-treated rNSphs might explain the higher oligodendrogenic response to GFW compared to non-treated rNSphs. In conclusion, these data announce that soluble factors derived from MSCs prime proliferating rNSphs to oligodendrogenesis.

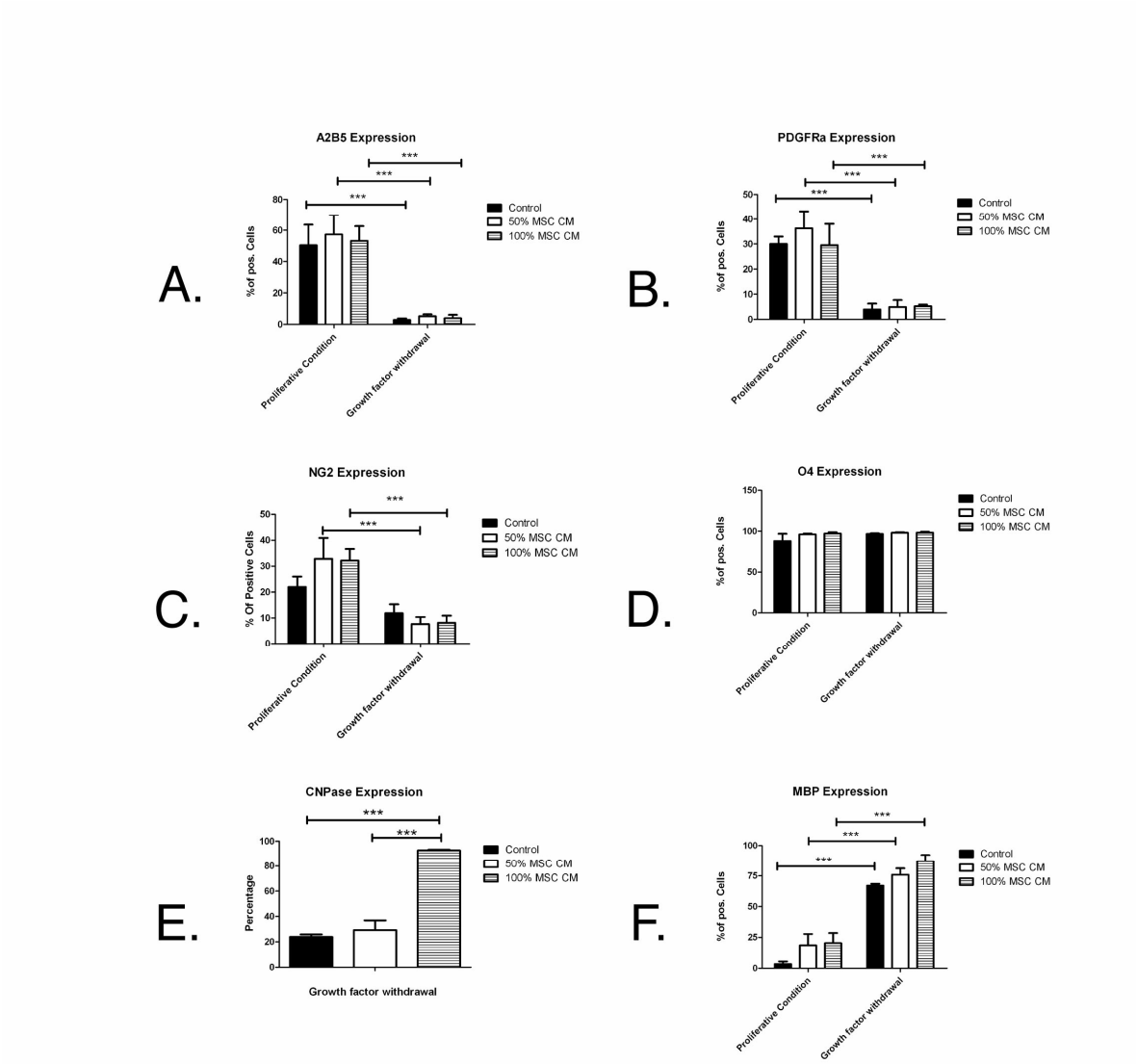


Figure 8. Effects of MSC-CM on the oligodendrogenic capacity of proliferating rNSphs.
Pre-incubated rNSphs were dissociated and seeded overnight under serum-free conditions. Then cells were either fixed to analyze the marker expression profile under proliferative conditions or either were incubated in the absence of growth factors up to 7 days and fixed to analyze the growth factor response. Immunofluorescence was performed for the different markers of the oligodendrogenic progression (early and middle and late). Quantitative marker expression of A2B5 **(A)**, PDGFRα **(B)**, NG2 **(C)**, O4 **(D)**, CNPase **(E)** and MBP **(F)**. Note that after growth factor withdrawal cells showed a decrease in the expression of oligodendrocyte progenitor markers (A2B5, PDGFRa, NG2) and an increase in the expression of the mature oligodendrocyte marker MBP. Observe that rNSphs pre-incubated with MSC-CM display more MBP- and CNPase- expressing cells than control conditions after growth factor withdrawal. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

IV. 2. Effects of MSC-CM on proliferating mNSphs

IV.2.1. MSC-CM promotes proliferating mNSphs adhesion affecting cell morphology

In a second part of experiments we wanted to examine the effect of soluble factors, driven from MSCs on proliferating mNSphs. mNSphs have different properties in attachment, response to growth-factors etc. compared to rNSphs as mentioned in the introduction before (Ray et. al. 2006). For the following experiments MSC-CM was used again as described in III.2.5. mNSphs were incubated under normal proliferating conditions (control), 50% MSC-CM or 100% MSC-CM. After 7, 14 or 21 days cell morphology was analyzed at the Olympus microscope.

Proliferating mNSphs grew mainly attached on the plate, only some mNSphs grew as floating aggregates, when the cells grew under normal proliferation conditions (control) (figure 9 A, D, G). Proliferating mNSphs, exposed to 50% MSC-CM and 100% MSC-CM displayed a less sphere formation than in the control condition, independent of incubation time (figure 9 B, C, E, F, H, I).

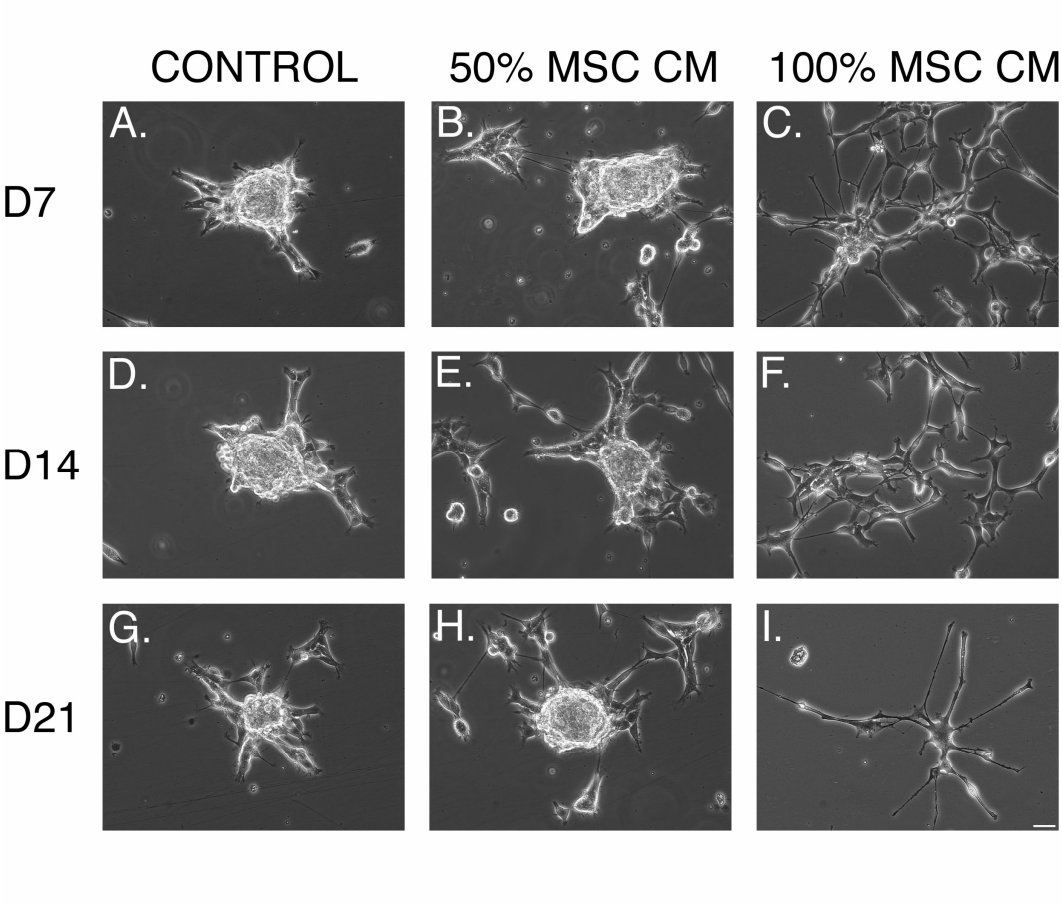


Figure 9. Morphology of proliferating mNSphs.
Phase contrast images of mNSphs in normal proliferation medium (control) **(A,D,G)**; treated with 50% MSC-CM medium **(B,E,H)** or with 100% MSC-CM **(C,F,I)**. Proliferating mNSphs were incubated during 7 days **(A-C)**, 14 days **(D-F)** and 21 days **(G-I)**. Note that independent of the incubation time proliferating mNSphs exposed to 100% MSC-CM displayed less sphere formation than control condition. Scale bar = 100 µm

IV.2.2 MSC-CM treatment decreases the absolute number of mNSphs in vitro

As mentioned above mNSphs displayed a less sphere formation under treatment with 50% MSC-CM and 100% MSC-CM. To check if these decrease in sphere formation was caused by a decrease of cell number, mNSphs were incubated under normal proliferating conditions (control), 50% MSC-CM or 100% MSC-CM. After 7, 14 and 21 days cell number was analyzed by Trypan blue exclusion.

As expected the percentage of 50% MSC-CM and 100% MSC-CM treated cells was significant lower compared to the control, 21 days after seeding (approximately 90% figure 10 A, B).

The obtained growth curve showed the difference of the growth rate of MSC-CM and not MSC-CM treated cells. Not treated control cells showed an exponential growth

curve, whereas 50% MSC-CM and 100% MSC-CM showed a final plateau, after a small expansion phase.

In summary these data exhibited that treatment with 50% MSC-CM and 100% MSC-CM decreases the absolute cell number of proliferating mNSphs.

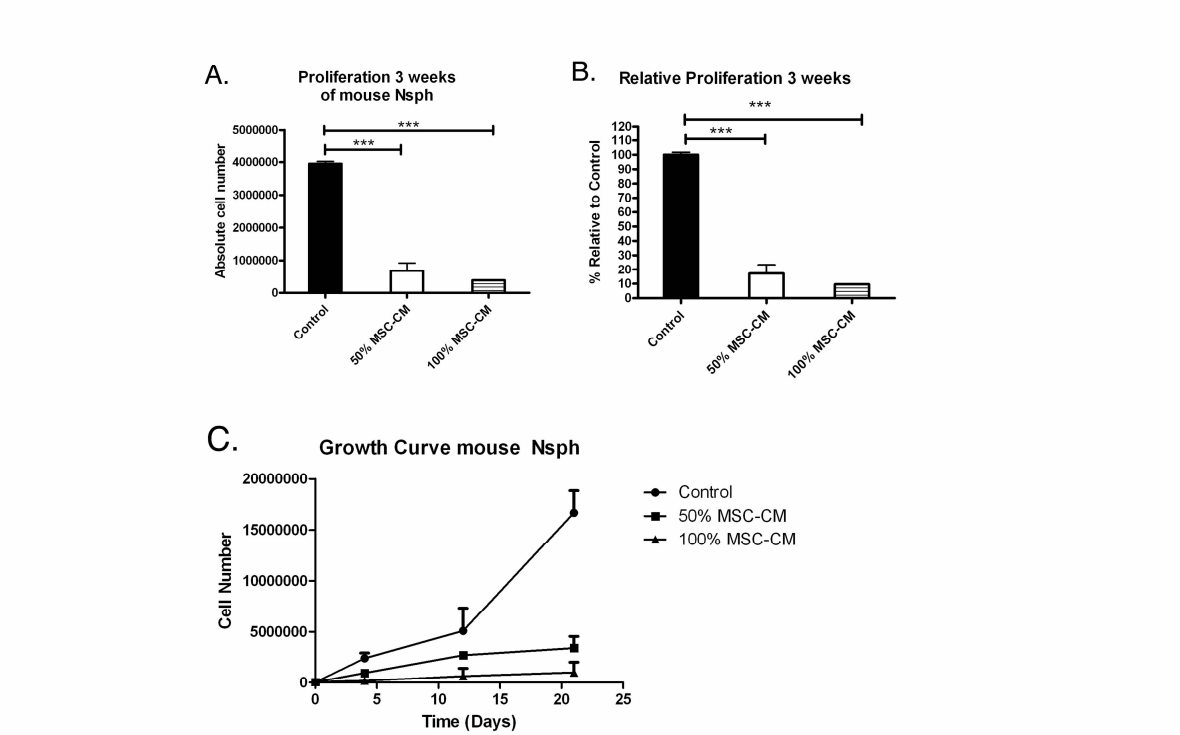


Figure 10. MSC-CM treatment decreases the absolute cell-number of mNSphs in vitro.
mNSphs were incubated in normal proliferation medium (control), 50% MSC-CM and 100% MSC- CM, to check the influence of MSC-CM on the proliferation of proliferating mNSphs. Cell number was analyzed by Trypanblue exclusion after 7, 14 and 21 days. Note that treatment with MSC-CM decreases the absolute cell number around 80% in 50% MSC-CM and 100% MSC-CM compared to the control **(A, B)**. After a small expansion phase 50% MSC-CM and 100% MSC-CM treated cells show a final plateau, whereas the control group shows an exponential growth curve **(C)**.

IV.2.3 MSC-CM enhances the expression pattern of glial and progenitor markers of proliferating mNSphs

To determine whether MSC-CM affects the expression pattern of cell lineage specific markers, non-treated (control) and MSC-CM-treated proliferating mNSphs were dissociated. After this procedure single cells were seeded overnight under serum-free conditions to allow them to attach. The cells were fixed and marker expression was analyzed in the following.

Most of the cells carried from proliferating mNSphs expressed the glial progenitor markers A2B5 (approximately 25% figure 11 C, D) and NG2 (approximately 20% figure 11 E, F) and the stem cell marker Nestin (approximately 20% figure 11 A, B).

MSC-CM-treated proliferating mNSphs displayed a significant increase in the percentage of oligodendroglial progenitor markers NG2 and O4 (figure 11 E, F, G, H). However the percentage of A2B5 expressing cells was not chanced by treatment with MSC-CM. Further the MSC-CM-treated cells showed a significant increase of GFAP/Nestin expressing cells (figure 11 A, B from approximately 10% in the control group to approximately 40% in the 100% MSC-CM). At least, a few number of cells expressed the neuronal progenitor marker DCX by treatment with 100% MSC-CM (less than 2% figure 11 I, J).

In summary these findings imply that soluble factors derived from MSCs increased the percentage of glial and oligodendroglial markers of proliferating mNSphs.

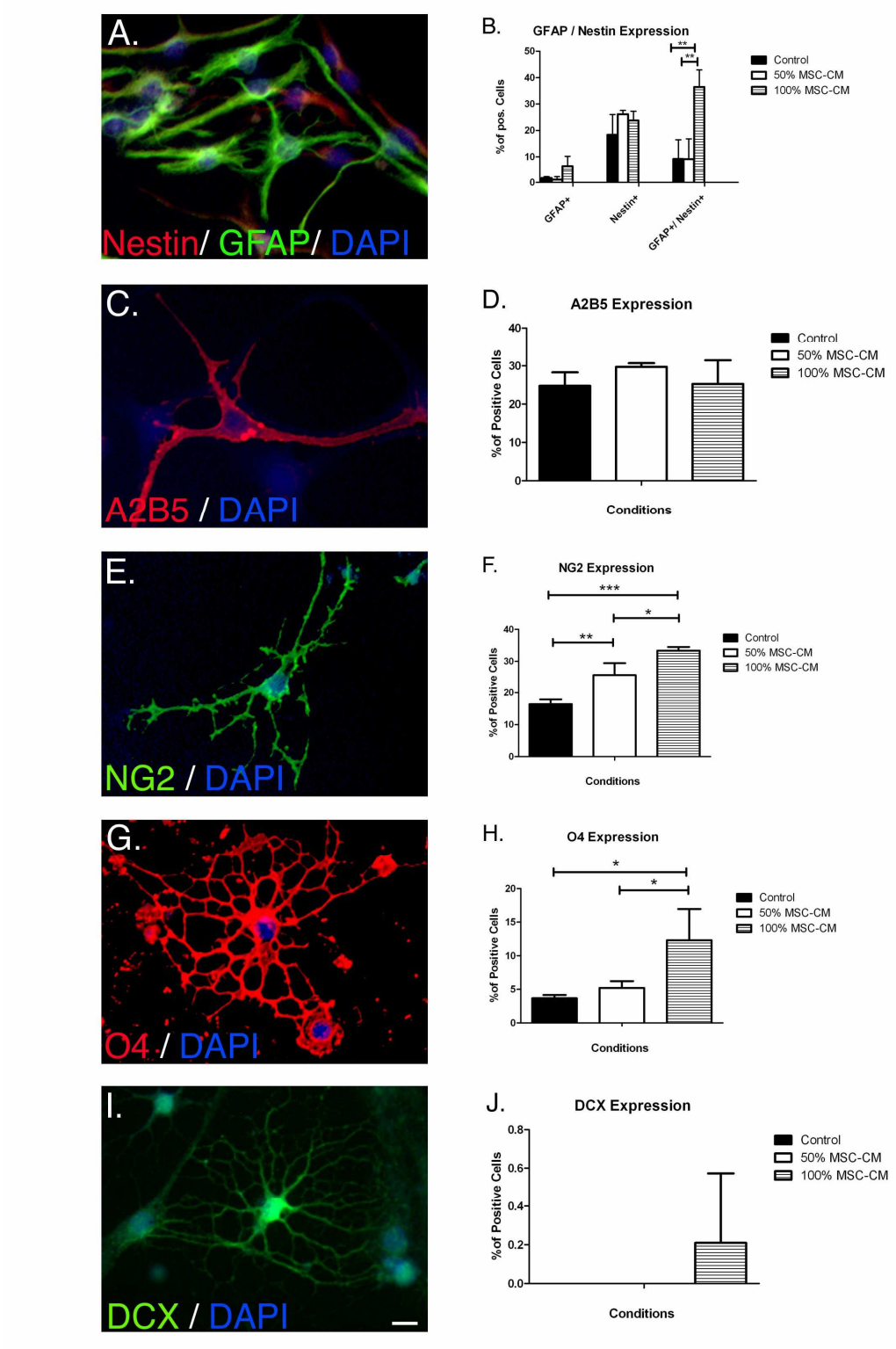


Figure 11. Marker expression profile of proliferating mNSphs pre-incubated 21 days under control conditions, 50% MSC-CM and 100% MSC-CM.
Treated mNSphs were dissociated and cells were seeded overnight under serum-free conditions and then fixed. Marker expression was analyzed by immunofluorescence. Illustrative fluorescence images are shown for Nestin (red), GFAP (green) and DAPI (blue) **(A)**; A2B5 (red) and DAPI (blue) **(C)**; NG2 (green) and DAPI (blue) **(E)**; O4 (red) and DAPI **(G)**; and DCX (green) and DAPI (blue) **(I)**. Quantitative analysis for each marker are shown (B, D, F, H and J). Note the significant increase of GFAP+/Nestin+ cells (neural stem cell phenotype) in 100% MSC CM, compared with control condition. Also note that MSC-CM significant increase the percentage of cells expressing oligodendroglial progenitor markers (NG2 and O4). * = p<0.05; ** = p<0.01; *** = p<0.001. Scale bar = 100µm

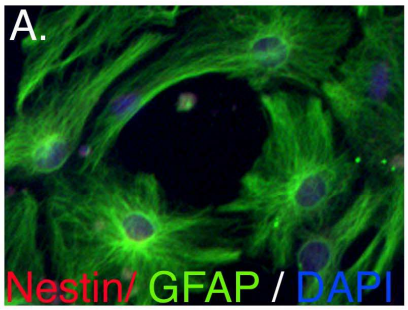
IV.2.4. MSC-CM enhances the expression of neural stem cell markers in proliferating mNSphs

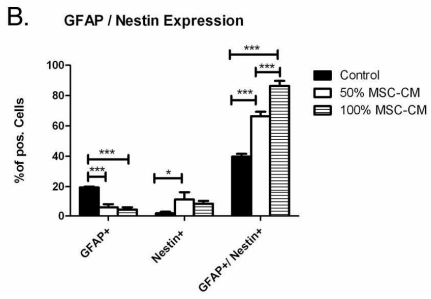
In a recent study it was shown that mNSphs under serum containing conditions differentiate predominantly into astrocytes after GFW. In addition, a minor subpopulation of mNSphs generated DCX-expressing neurons (Steffenhagen et al., 2011).

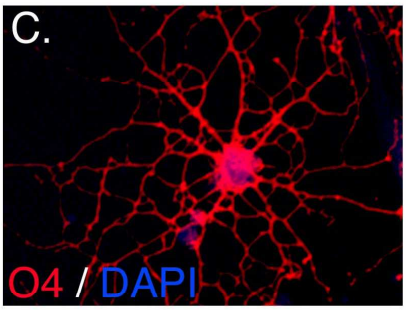
It is one aim of this thesis to clarify if adult mNSphs differentiate into astrocytes after GFW, when they were treated in serum-free conditions before. Furthermore to check whether MSC-CM has also an oligodendrogenic response on mNSphs after GFW, non-treated and MSC-CM-treated proliferating mNSphs were dissociated and seeded overnight under serum-free conditions and incubated for 7 days without growth factors (FGF-2 and EGF).

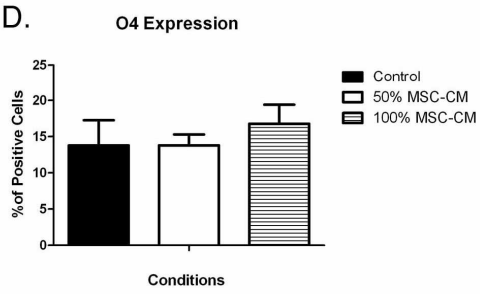
In contrast to the rNSphs, mNSphs showed a significant decrease in the percentage of the mature oligodendrogenic marker expression (MBP and CNPase) in the 50% MSC-CM and 100% MSC-CM treated group (figure 12 E, F, G, H). However MSC-CM displayed no influence on the O4 expression of mNSphs (figure 12 C, D).
Admittedly we found a significant increase in expression of the astroglial/ neural stem cell markers GFAP/ Nestin in the 100% MSC-CM treated cell group, whereas the percentage of GFAP single labelled cells was decreasing (figure 12 A, B).
Finally, there were few cells that express the neuronal progenitor markers DCX and MAP2ab in response to GFW (less than 4% figure 12 I, J, K, L) and MSC-CM treatment did not affect its expression (figure 12 J, L).

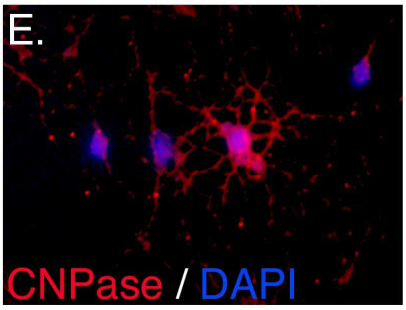
In summary, these data insinuated that MSC-CM has no effect on the oligodendrogenic ability of proliferating mNSphs on the one hand. But on the other hand MSC-CM may shift the cells towards an astrogenic / stem-cell fate like the increase of the astroglial/ neural stem cell marker GFAP/ Nestin let us suggest.

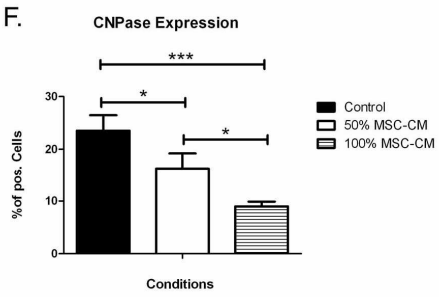

A.
Nestin/ GFAP / DAPI

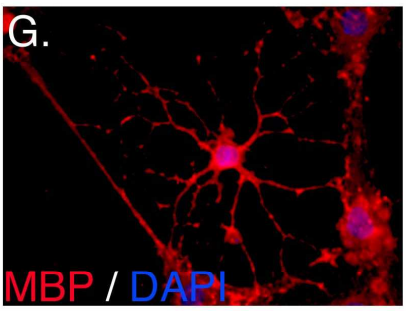

B.
GFAP / Nestin Expression
%of pos. Cells
Control
50% MSC-CM
100% MSC-CM
GFAP+
Nestin+
GFAP+/ Nestin+

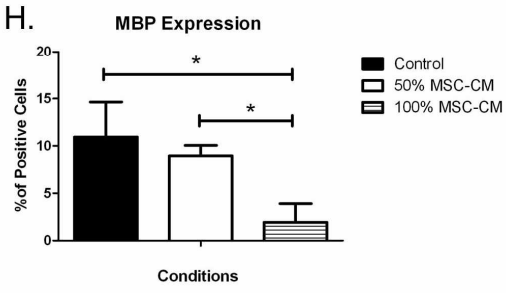

C.
O4 / DAPI

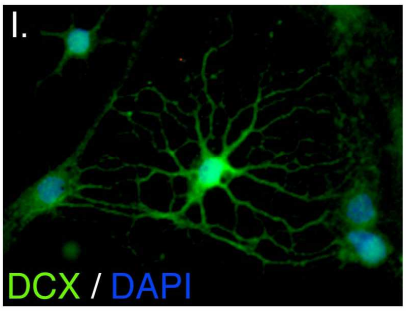

D.
O4 Expression
%of Positive Cells
Control
50% MSC-CM
100% MSC-CM
Conditions

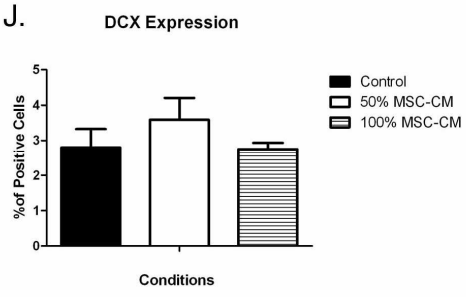

E.
CNPase / DAPI


F.
CNPase Expression
%of pos. Cells
Control
50% MSC-CM
100% MSC-CM
Conditions


G.
MBP / DAPI


H.
MBP Expression
%of Positive Cells
Control
50% MSC-CM
100% MSC-CM
Conditions


I.
DCX / DAPI


J.
DCX Expression
%of Positive Cells
Control
50% MSC-CM
100% MSC-CM
Conditions

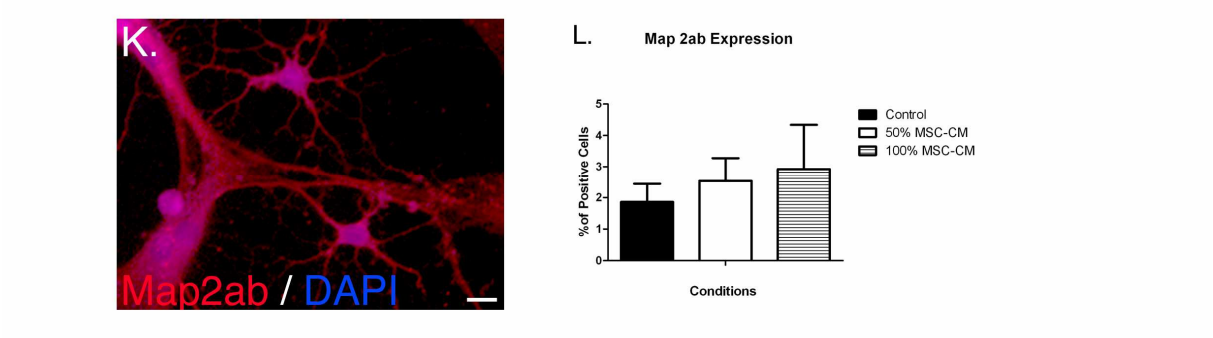


Figure 12. Growth factor withdrawal response of mNSphs pre-incubated 21 days under control condition, 50% MSC-CM and 100% MSC-CM.
Treated mNSphs were dissociated, seeded and incubated for one week under serum-free conditions without growth factors. After one week the cells were fixed and stained for immunohistochemistry. Marker expression was analyzed by immunofluorescence. Illustrative flourescence images are shown for: Nestin (red), GFAP (green) and DAPI (blue) **(A)**; O4 (red) and DAPI (blue) **(C)**; CNPase (red) and DAPI (blue) **(E)**; MBP (red) and DAPI **(G)**; DCX (green) and DAPI (blue) **(I)**; and MAP2ab (red) and DAPI (blue) **(K)**. Quantitative analysis for each marker are shown in the left panels **(B, D, F, H, J and L)**. Note the significant increase in the percentage of cells that express neural stem cell markers (GFAP+/Nestin+) when mNSphs were grown in 100% MSC-CM respect to control condition. Note also that MSC-CM decreases the percentage of cells that express the mature oligodendrocyte marker MBP. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. Scale bar = 100µm

IV.2.5 MSC-CM increases the rate of apoptosis of mNSphs and led the mNSphs to stem cells

As we mentioned before the absolute number of proliferating mNSphs was decreasing, when cells were incubated with 50% MSC-CM and 100% MSC-CM. To check, if these effect was caused through apoptosis or necrosis we performed a CytoTox96®Non-Radioactive Cytotoxicity Assay as described in III.2.10 7, 14 and 21 days after seeding.

We were able to show that 50% MSC-CM and 100%MSC-CM increase significant the LDH-activity, compared to the control (figure 13 A, B, C) both 7, 14 and 21 days after seeding, indicating that the anti-proliferative effect of MSC-CM on mNSphs is a toxic effect. Interestingly this cytotoxic effect was time-dependent. These data indicated that MSC-CM induces apoptosis or necrosis in mNSphs.

One possible mechanism for an improved cytotoxicity could be associated to changes of the cell cycle. We therefore investigated the effects of MSC-CM on the proliferation of mNSphs via FACS analysis 7 and 14 days after seeding as described in III.2.9. When the cells were incubated with 50% MSC-CM or 100% MSC-CM, 76%

respectively 52% of the cells were present in the G1/ S / G2M population, compared to 83% in the control group (figure 13 D, E, F). In contrast to these data cells, present in the G0 state increased from 17% in the control group to 24% respectively 48% in the 50% MSC-CM and 100%MSC-CM group. Similar to these data 14 days after seeding 85% of the control cells were in the G1/ S/ G2m state, whereas MSC-CM decreases these percentage to 73% in 50% MSC-CM respectively 6% in the 100% MSC-CM treated cells. In contrast to these data, the percentage of cells, which are present in the G0 state increased from 15% in the control group to 28% in the 50% MSC-CM treated group and 94% in the 100% MSC-CM treated group (figure 13 G, H, I). It seemed that treatment with MSC-CM led the cells in the G0-state of the cell-cycle.

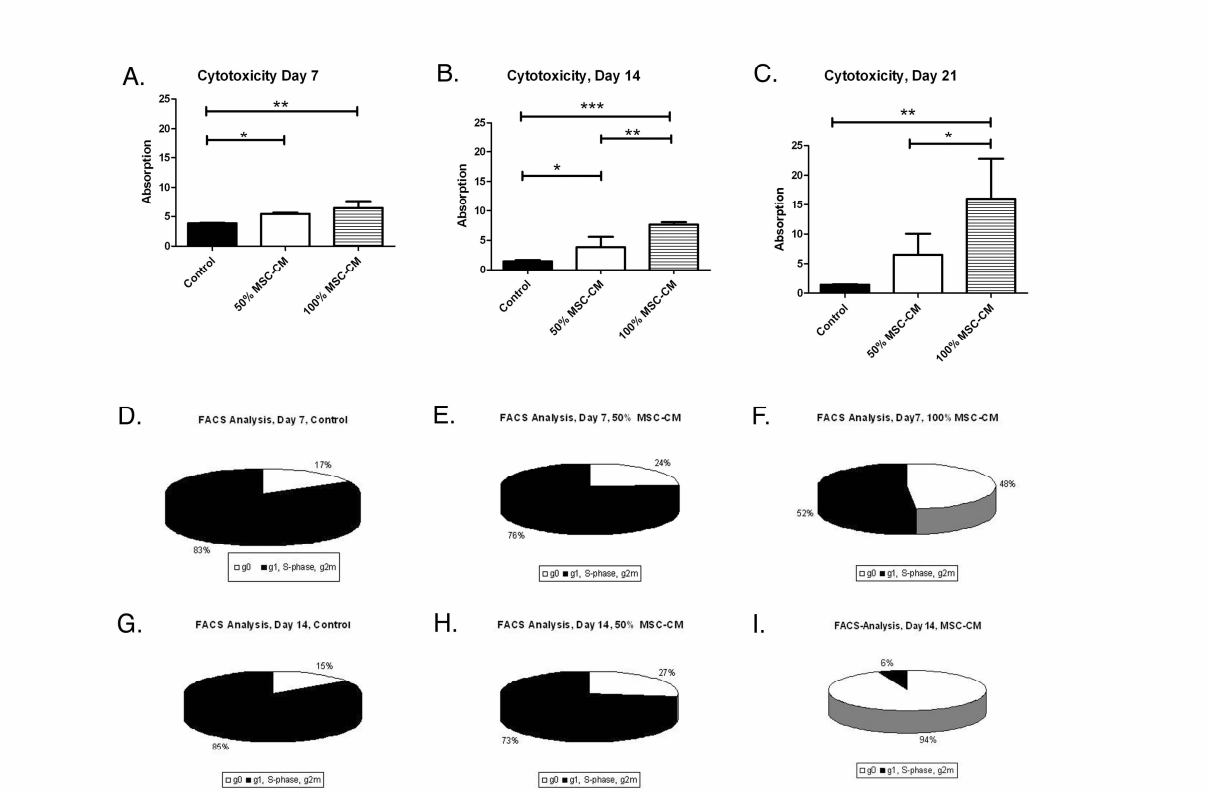


Figure 13. Treatment with MSC-CM induces apoptosis/ necrosis in proliferating mNSphs and led the cells to the G0- state of the cell- cycle.
mNSphs were cultured in normal proliferation medium (control), 50% MSC-CM and 100% MSC-CM for 7, 14, and 21 days. After the accordant number of days a CytoTox96®Non-Radioactive cytotoxicity assay was performed first **(A-C)**. Note the significant increase of LDH in the 50% MSC-CM and 100% MSC-CM treated group 7, 14 and 21 days after seeding.
In a second step of experiments the effect of MSC-CM on proliferating mNSphs was performed via Fluorescence-Activated Cell Sorter (FACS analysis) 7 and 14 days after seeding **(D-I)**. Note the increased percentage of cells in the G0 state, when cells were treated with 50% and 100% MSC-CM.

IV.2.6 MSC-CM enhances the expression of the markers of self renewal

After we saw the increase of cells in the G0 cell state, treated with 100% MSC-CM we aimed to analyse the expression pattern of markers which are known to show the self renewal potential of NSC. It's known that Bmi1 is necessary for NSC self-renewal and it represses the cell cycle inhibitors p16, p19 and p21 (Fasano et al. 2009), as far as it's known about the importance of Sox2 in the self renewal of NSC (Hu et al., 2010).

Therefore, proliferating mNSCs were incubated up to 3 weeks under normal proliferating conditions (control), 50% and 100% MSC-CM, the expression level of Bmi1 and Sox2 were analyzed by quantitative RT-PCR and the Bmi-1/ Sox2 ration was determined for the different conditions. The PCR revealed that the expression of Bmi1 and Sox2 was higher in the 50% MSC-CM and 100% MSC-CM treated group compared to the control, but not significant (figure 14 A, B, C). These data led us assume that MSC-CM enhance the self renewal potential of proliferating NSC.

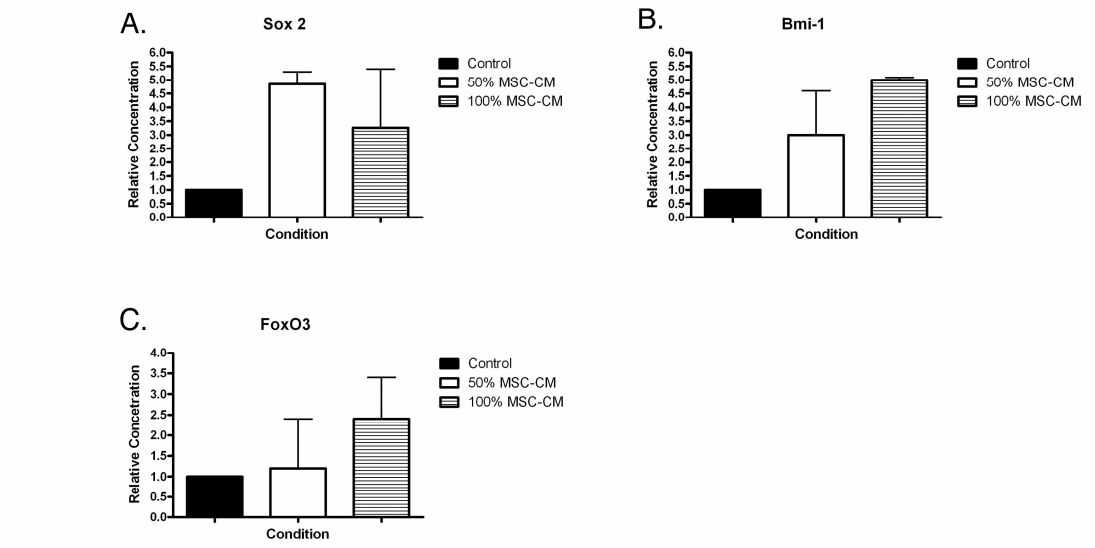


Figure 14.Treatment with MSC-CM enhances the expression of markers of cell self renewal. Cells were treated with normal proliferation medium (control), 50% MSC-CM and 100% MSC-CM for 21 days under normal proliferation conditions. The after 21 days performed PCR showed an increase in the percentage of the markers Bmi-1, Sox2 and FoxO3, which led us suggest that treatment with MSC-CM enhance the self-renewal potential of mNSphs **(A-C)**.

V. Discussion

This thesis aimed to characterise the different effects of MSC-CM on adult proliferating rat and mouse neural hippocampal stem cells.

The studies and results of this thesis demonstrated that soluble factors derived from MSCs activated an oligodendrogenic program (OPr) and promoted the pre-disposition of proliferating adult rNSPCs towards an oligodendrocyte fate. In contrast to these investigations proliferating adult mNSPCs displayed no pre-disposition towards an oligodendrocyte fate, but MSC-CM shifted them towards an astroglial lineage and may have increased the mNSPCs stemness.

V.1 MSC-CM primes the oligodendrogenic program in proliferating adult rNSCs

Over the last years several research groups studied the influence of different stimuli on proliferating and differentiating adult NSCs. Adult hippocampal NSCs, as used in this thesis showed the main characteristics of NSCs – named the ability of self-renewal and the generation of both glia and neurons *in vitro* and *in vivo* (Palmer et al., 1997; Boda and Buffo, 2010).
At first these NSCs can be encouraged to proliferate, which means to maintain the stem cell character. They can further be provided to differentiate into mature neural cell types via several steps by withdrawing mitogenic growth factors and / or adding specific factors promoting differentiation into particular neural lineages. A neuronal differentiation can be reached through retinoic acid (RA), Platelet derived growth factor – AA (PDGF – AA), PDGF – AB, PDGF - BB and neurotrophin-3 (NT- 3) (Johe et al., 1996). Ciliary neurotrophic factor (CNTF), Leukemia inhibitory factor (LIF) and bone morphogenetic protein-2 and -4 (BMP-2/-4) induce astrocytic differentiation (Mayer et al., 1994; Johe et al., 1996; Cheng et al., 2007) while insulin-like growth factor-1 (IGF-1) and mesenchymal stem cell conditioned media (MSC-CM) induce oligodendroglial differentiation (Hsieh et al., 2004; Rivera et al., 2006). The thyroid hormone T-3 influences the differentiation of multipotent precursors of the adult rat brain toward a mixed glial fate (Johe et al., 1996). It is apparent from previous studies

that four distinctly different subsets of ligand-receptor superfamilies are involved in determining the fate of adult NSCs. These include 1) the transforming growth factor type-beta-1 (TGF-beta1) and bone morphogenetic protein (BMP) superfamily; 2) the platelet-derived and epidermal (PDGF/EGF) growth factors; 3) the interleukin-6, leukemia inhibitory factor, and ciliary neurotrophic factor (IL-6/LIF/CNTF) superfamily; and 4) the EGF-like Notch/Delta group of extracellular ligands (Mondal et al., 2004).

Although lineage specifications can be reached through GFW, it seems that cell priming as a well as cell fate and lineage decisions are taken while progenitors are still proliferating (Rivera et al., 2010). For example, PDGFα in cooperation with bFGF converts embryonic derived proliferating neurospheres into oligospheres, which consist of oligodendrocyte precursor cells (Pedraza et al. 2008). Transient exposure to lithium chloride during *in vitro* proliferation has the ability to alter the differential fate of rNSCs *in vitro* and increase the proportion of cells expressing neuronal markers while at the same time causing a reduction in glial progeny (Vazey and Connor, 2009). A mixture of FGF, heparin and laminin was further shown to prime adult human neuronal progenitors towards neurons (Olstorn et al., 2011). For instead the data of this thesis demonstrated that MSCs prime proliferating NSCs, reinforcing cell fate decision and accelerating differentiation towards oligodendrocytes.

However other data indicate that cell fate decision might also occur after cell-cycle exit, when cells are differentiating. Indeed, it was shown that it is not sufficient to force spinal cord derived neural progenitor cells to the cell-cycle to alter the cell fate decision of these cells (Lobjois et al., 2008). Therefore, cell fate decisions and cell progenitor priming might represent different independent biological events. While the first could occur independently of cell cycle exit and involved the cell lineage determination, the second might particularly take place in proliferating progenitors and consist on a pre-disposition induction towards a certain cell lineage that boost the fate choice and ends up on a faster differentiation.

In the present work, it was described that soluble factors derived from MSCs promote the pre-disposition of proliferating adult rNSCs towards an oligodendrocyte fate. MSC – CM did not impinge on cell proliferation, nor did it modify the expression pattern of typical neural / oligodendroglial progenitor markers. However, it predisposed and / or accelerated progenitors to differentiate into mature MBP expressing oligodendrocytes and generated a higher amount of CNPase positive cells.

A surprising observation within the present work is the notion that the majority of progenitors (approximately 60%) that were generated under control conditions differentiated into MBP expressing oligodendrocytes in serum-free conditions. For instance, after GFW rNSCs differentiated into MBP-expressing adult oligodendrocytes, which was enhanced by MSC-CM (Steffenhagen, et al., 2011). This suggests that the progenitors used in the present study where already partly committed to an oligodendroglial fate. This tendency to generate oligodendrocytes might not have been minded in many previous studies, since most of the differentiation assays are typically performed in serum-containing, and thus astrocyte-inducing, media.

Little is known about the molecular mechanism how MSC-CM induces an oligodendroglial activity. During the NSCs differentiation, cells change their internal program from the self-renewal state to a committed fate. When differentiation is activated, specific cell fate genes are up-regulated by activator markers, while repressor markers are lost (Azuara et al., 2006 ; Spivakov et al., 2007; Gaspar-Maia et al., 2009; Bernstein et al., 2006). It seems that the effect of MSC-CM involves the regulation of Olig and Id proteins. Olig1 and Olig2 play an important role in oligodendroglial fate determination of neural progenitors during the development of the CNS as well as during oligodendrogenesis in the adulthood (Ross et al., 2003). Their inhibition leads to the abolishment of oligodendrocyte differentiation (Zhou and Anderson, 2002). The inhibitor of differentiation 2 (Id2) and 4 (Id4) proteins interact with Olig1 and Olig2 by sequestration of the Olig factors and their binding partners E12 and E47 so that the Olig-E complex cannot be formed and its entry into the nucleus is prevented (Wang et al., 2001). Therefore, oligodendroglial fate determination of neural progenitor cells is inhibited and astrocytic differentiation encouraged. That is why the ratio of the amount of Olig and Id2 proteins is a key determinant for oligodendrocyte versus astrocyte fate decision (Samanta and Kessler, 2004). In a previous study, it was already demonstrated that MSC-CM induces Olig2 and reduces Id2 expression in neural progenitors that underwent serum-induced differentiation (Rivera et al., 2006). Under proliferation conditions, MSC-CM did not increase Olig2 expression, but constrained the expression of Id2 and thus raised the Olig2/Id2 ratio. As a consequence, the Id2 appears to be a key regulator in the process. Since Id2 is up-regulated by BMPs signalling (Samanta and Kessler, 2004), MSC-CM might block BMPs signalling in order to decrease Id2 expression. Along this line, the BMPs an-

tagonist noggin induces oligodendrogenesis of human embryonic progenitors (Izrael et al., 2007) suggesting noggin as a candidate factor present in the MSC-CM. However, in our previous work we excluded noggin as the oligodendrogenic activity present in MSC-CM (Rivera et al., 2008). Nevertheless, the possibility that MSC-CM activate a signal transduction pathway that decreases Ids expression suggests a link or crosstalk to BMP signalling.

In conclusion, MSCs exert a potent oligodendrogenic effect on adult rat NSCs promoting and enhancing cell fate decision, differentiation and maturation. Both, the identification of the MSCs derived oligodendrogenic activity as well as the clarification of the molecular mechanism underlie are crucial to develop new therapies for the treatment of demyelinating diseases. Demyelinating diseases, such as multiple sclerosis (MS) or spinal chord injury, are characterized by the loss of oligodendrocytes resulting in several neurological symptoms including paralysis. Enhancing oligodendrogenesis and promoting remyelination might be an attractive approach to counteract this situation. The feasibility of this approach is provided by the fact that oligodendrogenesis occurs during adulthood, where neural and oligodendroglial progenitors are the cellular source of remyelinating cells.

V.2 MSC-CM enhances the expression of astrocyte / stem cells markers on proliferating adult mNSCs

In a second trial of experiments the effects of soluble factors derived from MSCs on proliferating mNSCs have been explored. In previous studies it was shown that adult mNSCs strongly express Nestin, used as stem cell marker, and GFAP, used as marker for astrocytes (Steffenhagen et al., 2011).

Coincident with these observations an astroglial/ stem cell marker expression of proliferating mNSCs was detected, which gave rise mainly to astrocytes as well as to some neurons after GFW. After the treatment with MSC-CM the expression of GFAP/Nestin was significantly enhanced in proliferating mNSCs. On the base of these observations it was hypothesized that MSC-CM could proliferating mNSCs shift towards an astroglial/ stem cell identity.

On this point someone could argue, based on the increase on the proportion of Nestin- and GFAP-expressing cells, that mNSCs are more stem cell-like than rNSCs and MSC-CM may enhance the stemness of proliferating mNSCs. But actually, there are no markers that are specific for adult NSCs. Nestin and GFAP are markers for precursors and astrocytes. However radial glia cells and astrocytes are considered to be the NSCs in the SGZ of the dentate gyrus, called type-1 cells (Seri et al., 2001). Type-1 cells express the astrocyte marker GFAP as well as Nestin and Sox2. They give rise to type-2 cells, which are highly proliferative progenitor cells and can be differentiated into two subtypes: DCX-negative type-2a cells and DCX-expressing type-2b cells. Type-2a cells generate type-3 cells, which represent the migrating neuroblasts and differentiate into mature neurons (Kempermann et al., 2004).
Although mNSCs display a stem cell identity by the expression of GFAP / Nestin they are no true stem cells, because they don't show the main characteristics of stem cells. Multipotency and the potential of self-renewal are these main characteristics of stem cells. In a previous study Steffenhagen showed that mNSCs display a restricted differentiation potential (Steffenhagen et al., 2011). After GFW as well as after additional treatment with neurogenic, astrogenic and oligodendrogenic stimuli, namely RA, BMPs and MSC-CM, mNSCs gave rise to astrocytes and some neurons but they could not generate oligodendrocytes (Steffenhagen et al., 2011). So it was concluded that mNSCs display an intrinsic astrogenic fate potential and were shifted back to an astroglial / stem cell fate by MSC-CM. To verify the true stem cell character of these MSC-CM treated cells more experimental approach is necessary to demonstrate the multipotency as well as the self-renewal capacity of these cells.

Although the cellular mechanisms that underlie the MSC-CM impact of proliferating mNSCs remain unclear, it might imply cell proliferation and cell death. Thus, together with a reduction in the proliferation rate of mNSCs after treatment with MSC-CM, an elevated rate of cell death was observed.
First, the anti-proliferative MSC-CM activity on mNSCs might involve the cell cycle. As described before the residual mNSCs after the treatment with MSC-CM displayed a significant reduction in cell proliferation as well as elevated levels of the astroglial / stem cell markers GFAP/Nestin. There it was hypothesized that MSC-CM increases the stemness of proliferating adult mNSCs by cell cycle modification.

The cell cycle consists of four distinct phases: G_1 phase, S phase (synthesis), G_2 phase (collectively known as interphase) and M phase (mitosis). In the G_0 state, the cell has left the cycle and has stopped dividing (cell cycle arrest). These cells are stem cells or differentiated cells normally. The experiment of this thesis demonstrated that MSC-CM leads mNSCs towards a cell cycle arrest, so that the mNSCs were more quiescent. This means that MSC-CM promotes either stem cells or led the adult mNSCs differentiate into astrocytes. Although stem cells exhibit a slower proliferation rate than progenitors, as this thesis sowed and although the remaining cells exhibit the astroglial / stem cell markers GFAP/Nestin it remains unclear if these cells are true stem cells or if they just display few stem cell features.

Second, a MSC-CM cytotoxic effect on proliferating mNSCs was also found by increased LDH levels. LDH is an intracellular enzyme present in a wide variety of organisms, including plants and animals. Given that the LDH is an intracellular enzyme, a higher amount of the measurable extracellular LDH is an indicator for cell death. Cell death can occur through apoptosis and necrosis. Apoptosis is the process of programmed cell death. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis differs from necrosis, which is a form of traumatic cell death that results from acute cellular injury. After necrosis the cellular debris can damage other cells of the organism. The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide or cytokines (Popov et al., 2002; Brüne, 2003). Although a cytotoxic impact of MSC-CM on proliferating mNSCs through elevated LDH-levels was attested, it is not possible to distinguish if MSC-CM dispatch mNSCs randomly or selectively nor it can be distinguished if MSC-CM activates the process of the programmed cell death (apoptosis). It remains also unclear if MSC-CM has an inductive effect on a minor astrocyte/stem cell subpopulation of proliferating mNSCs. It would have to be demonstrated if MSC-CM has a selective or an inductive impact on proliferating mNSCs through pulse-chase experiments to determine the propidium iodide (PI) incorporation and staining of the different cell subpopulations. PI is an intercalating agent and a fluorescent molecule that can be used

to stain DNA (Sakimoto et al., 2006). It can be used to differentiate necrotic, apoptotic and normal cells (Lecoeur 2002).

To demonstrate the real stem cell character of these remaining cells after treatment with MSC-CM, the two main stem cell criteria must be verified: multipotency and self-renewal. Multipotency can be evidenced if these cells can differentiate into neurons, astrocytes and oligodendrocytes after the treatment with neurogenic, astrogenic and oligodendrogenic stimuli. If the remaining cells are still able to self-renew, it can be demonstrated through a secondary neurosphere assay. For that, cells from the primary NSph culture are dissociated and single cells are plated to explore whether these single cells are able to form secondary NSph.

The molecular mechanism of the MSC-CM induced astroglial/ stem-cell activity isn't identified yet. Multiple cell-intrinsic regulators coordinate with the microenvironment through various signalling pathways the regulation of adult mNSCs cell maintenance, self-renewal, and fate determination. These regulators include the nuclear hormone receptor TLX (tailless), the high-mobility-group transcription factor Sox2 (sex determining region of Y chromosome-related high mobility group box 2), the basic helix-loop-helix (bHLH) transcriptional repressor Hes (hairy and enhancer of split), the tumour suppressor phosphatase Pten (phosphatase and tensin homolog deleted on chromosome 10), and the Drosophila membrane-associated protein Numb homologs, Numb and Numblike (Qu and Shi, 2009). The Sox2 transcription factor is also expressed at high levels in neural stem and progenitor cells in both embryonic and adult brains (Episkopou, 2005). Along with Sox1 and Sox3, two other members of the Sox family, Sox2 has been shown to maintain neural stem cell identity in the developing brain. Constitutive expression of Sox2 inhibited neuronal differentiation and maintained neural progenitor characteristics, whereas inhibition of Sox2 led to precocious neuronal differentiation (Bylund et al., 2003; Graham et al., 2003). The polycomb family transcriptional repressor Bmi-1 is required for the self-renewal and post-natal maintenance neural stem cells from the CNS and PNS (Molofsky et al. 2003). Through further experiments we showed that MSC-CM involves the regulation of Bmi and Sox proteins in proliferating mNSCs. Through the elevated levels of these transcription factors we hypothesized that MSC-CM promotes self-renewal in proliferating mNSCs. Although Sox-2 and Bmi-1 are possible marker for self renewal, a

main characteristic of stem cells, the elevated levels of markers are not enough to identify stem cells.

In summary, it looks like that MSC-CM could induce an astrogenic / stem-cell fate on proliferating adult mNSCs. However the molecular pathways to reach this astrogenic/ stem-cell fate decision are not clear in detail, it seems that Bmi-1 and Sox2 play an important role. It is moreover unclear if MSC-CM has a de-differentiative process or a selective effect just as MSC-CM looks to be cytotoxic to mNSCs. To clarify this issue if the effect of MSC-CM is a de-differentiation process or a selection, more experimental approach is necessary. However the stem-cell potential of these residual cells must be evaluated to confirm the multipotency and the possibility of self-renewal of these cells.

V.3 In vivo clinical relevance of MSCs and NPCs interaction

Although a lot of key facts are poorly understood yet, such reports have fuelled expectations for the clinical exploitation of neural stem cells in cell replacement or recruitment strategies for the treatment of a variety of human neurological conditions including Parkinson's disease, Huntington's disease, multiple sclerosis and ischemic brain injury. Especially our finding that MSCs prime proliferating rNSC towards an oligodendroglial fate is of huge relevance in the treatment of demyelinating diseases since proliferating progenitors commonly surround CNS lesions in high numbers, but the processes of migration, differentiation and maturation in myelinating cells are defective in the case of these demyelinating diseases (Williams et al., 2007). It looks like that this lack of myelin-promoting activities might contribute to the impairment of remyelinisation in MS, as an example for demyelinating diseases. Searching the reason for this lack of myelin-promoting activities in MS, aging was detected as a possible risk factor, since endogenous remyelination is drastically affected in aged subjects. This age related decrease in remyelination is due to a deficient OPCs recruitment and differentiation (Sim et al., 2002). Thus, while remyelination impairment in MS and in particular during aging is partly derived from the failure to differentiate into oligodendrocytes, a lack of sufficient oligodendroglial commitment in proliferating progenitors might contribute to this deficiency. Therefore, the fact that MSCs not only induce an oligodendrocyte differentiation (Rivera et al., 2006) but also prime prolifer-

ating NPCs towards an oligodendrocyte fate might have *in vivo* and clinical relevance when aiming to develop remyelinating therapies for MS in young as well as in old patients.

Moreover neural stem cells may also find potential clinical application as cellular vectors for widespread gene delivery and the expression of therapeutic proteins through their ability of migration (Ostenfeld and Svendsen, 2003).

Since large numbers of stem cells can be generated efficiently in culture, they may obviate some of the technical and ethical limitations associated with the use of fresh (primary) embryonic neural tissue in current transplantation strategies. It's of huge relevance to get multipotent stem cells from non embryonic stem cells. Our findings that MSCs increase the stemness in proliferating mNSCs show the possibility to induce a stem cell fate from proliferating progenitors.

While considerable recent progress has been made in terms of developing new techniques allowing for the long-term culture of adult NSCs, the successful clinical application of these cells is presently limited by the understanding of both (1) the intrinsic and extrinsic regulators of stem cell proliferation and (2) those factors controlling cell lineage determination and differentiation. Although such adult NSCs may also provide accessible model systems for studying neural development, progress in the field has been further limited by the lack of suitable markers needed for the identification and selection of precursor and stem cells. There is a further need to distinguish between the committed fate (defined during normal development) and the potential specification (implying flexibility of fate through manipulation of its environment) of stem cells undergoing differentiation. With these challenges lying ahead stem-cell therapies are likely to remain within the experimental arena for the foreseeable future.

VI. References

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973.

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts. Proc. Natl. Acad. Sci. U.S.A 95:3908–3913.

Azuara V (2006) Profiling of DNA replication timing in unsynchronized cell populations. Nat Protoc 1:2171–2177.

Bai L, Caplan A, Lennon D, Miller RH (2007) Human mesenchymal stem cells signals regulate neural stem cell fate. Neurochem. Res 32:353–362.

Bai L, Lennon DP, Eaton V, Maier K, Caplan AI, Miller SD, Miller RH (2009) Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. Glia 57:1192–1203.

Bani-Yaghoub M, Tremblay RG, Lei JX, Zhang D, Zurakowski B, Sandhu JK, Smith B, Ribecco-Lutkiewicz M, Kennedy J, Walker PR, Sikorska M (2006) Role of Sox2 in the development of the mouse neocortex. Dev. Biol 295:52–66.

Barhum Y, Gai-Castro S, Bahat-Stromza M, Barzilay R, Melamed E, Offen D (2010) Intracerebroventricular transplantation of human mesenchymal stem cells induced to secrete neurotrophic factors attenuates clinical symptoms in a mouse model of multiple sclerosis. J. Mol. Neurosci 41:129–137.

Basak O, Taylor V (2009) Stem cells of the adult mammalian brain and their niche. Cell. Mol. Life Sci 66:1057–1072.

Bernhardt R, Matus A (1984) Light and electron microscopic studies of the distribution of microtubule-associated protein 2 in rat brain: a difference between dendritic and axonal cytoskeletons. J. Comp. Neurol 226:203–221.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES

(2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326.

Birkenkamp KU, Coffer PJ (2003) Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. Biochem. Soc. Trans 31:292–297.

Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL (1999) Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science 283:534–537.

Bobis S, Jarocha D, Majka M (2006) Mesenchymal stem cells: characteristics and clinical applications. Folia Histochem. Cytobiol 44:215–230.

Boda E, Buffo A (2010) Glial cells in non-germinal territories: insights into their stem/progenitor properties in the intact and injured nervous tissue. Arch Ital Biol 148:119–136.

Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J. Comp. Neurol 467:1–10.

Brüne B (2003) Nitric oxide: NO apoptosis or turning it ON? Cell Death Differ 10:864–869.

Bylund M, Andersson E, Novitch BG, Muhr J (2003) Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat. Neurosci 6:1162–1168.

Caccamo D, Katsetos CD, Herman MM, Frankfurter A, Collins VP, Rubinstein LJ (1989) Immunohistochemistry of a spontaneous murine ovarian teratoma with neuroepithelial differentiation. Neuron-associated beta-tubulin as a marker for primitive neuroepithelium. Lab. Invest 60:390–398.

Cameron HA, McKay R (1998) Stem cells and neurogenesis in the adult brain. Curr. Opin. Neurobiol 8:677–680.

Carlén M, Cassidy RM, Brismar H, Smith GA, Enquist LW, Frisén J (2002) Functional integration of adult-born neurons. Curr. Biol 12:606–608.

Carleton A, Petreanu LT, Lansford R, Alvarez-Buylla A, Lledo P (2003) Becoming a new neuron in the adult olfactory bulb. Nat. Neurosci 6:507–518.

Castro F de, Bribián A (2005) The molecular orchestra of the migration of oligodendrocyte precursors during development. Brain Res. Brain Res. Rev 49:227–241.

Cheng X, Wang Y, He Q, Qiu M, Whittemore SR, Cao Q (2007) Bone morphogenetic protein signaling and olig1/2 interact to regulate the differentiation and maturation of adult oligodendrocyte precursor cells. Stem Cells 25:3204–3214.

Chiacchiera F, Simone C (2010) The AMPK-FoxO3A axis as a target for cancer treatment. Cell Cycle 9:1091–1096.

Delpech B, Delpech A, Vidard MN, Girard N, Tayot J, Clement JC, Creissard P (1978) Glial fibrillary acidic protein in tumours of the nervous system. Br. J. Cancer 37:33–40.

Deng W, Obrocka M, Fischer I, Prockop DJ (2001) In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem. Biophys. Res. Commun 282:148–152.

Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H (2001) Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. Eur. J. Neurosci 14:1771–1776.

Dharmasaroja P (2009) Bone marrow-derived mesenchymal stem cells for the treatment of ischemic stroke. J Clin Neurosci 16:12–20.

Doetsch F, Scharff C (2001) Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav. Evol 58:306–322.

Episkopou V (2005) SOX2 functions in adult neural stem cells. Trends Neurosci 28:219–221.

Eroglu C, Barres BA (2010) Regulation of synaptic connectivity by glia. Nature 468:223–231.

Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S (2007) shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. Cell Stem Cell 1:87–99.

Fasano CA, Phoenix TN, Kokovay E, Lowry N, Elkabetz Y, Dimos JT, Lemischka IR, Studer L, Temple S (2009) Bmi-1 cooperates with Foxg1 to maintain neural stem cell self-renewal in the forebrain. Genes Dev 23:561–574.

Feng R, Rampon C, Tang YP, Shrom D, Jin J, Kyin M, Sopher B, Miller MW, Ware CB, Martin GM, Kim SH, Langdon RB, Sisodia SS, Tsien JZ (2001) Deficient neurogenesis in forebrain-specific presenilin-1 knockout mice is associated with reduced clearance of hippocampal memory traces. Neuron 32:911–926.

Furukawa-Hibi Y, Kobayashi Y, Chen C, Motoyama N FOXO transcription factors in cell-cycle regulation and the response to oxidative stress. Antioxid. Redox Signal 7:752–760.

Gage F, Kempermann G, Song H (2008) Adult neurogenesis. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press.

Gage FH, Ray J, Fisher LJ (1995) Isolation, characterization, and use of stem cells from the CNS. Annu. Rev. Neurosci 18:159–192.

Galli R, Borello U, Gritti A, Minasi MG, Bjornson C, Coletta M, Mora M, Angelis MG de, Fiocco R, Cossu G, Vescovi AL (2000) Skeletal myogenic potential of human and mouse neural stem cells. Nat. Neurosci 3:986–991.

Gaspar-Maia A, Alajem A, Polesso F, Sridharan R, Mason MJ, Heidersbach A, Ramalho-Santos J, McManus MT, Plath K, Meshorer E, Ramalho-Santos M (2009) Chd1 regulates open chromatin and pluripotency of embryonic stem cells. Nature 460:863–868.

Gerdoni E, Gallo B, Casazza S, Musio S, Bonanni I, Pedemonte E, Mantegazza R, Frassoni F, Mancardi G, Pedotti R, Uccelli A (2007) Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. Ann. Neurol 61:219–227.

Ghandour MS, Langley OK, Labourdette G, Vincendon G, Gombos G (1981) Specific and artefactual cellular localizations of S 100 protein: an astrocyte marker in rat cerebellum. Dev. Neurosci 4:66–78.

Gordon D, Pavlovska G, Glover CP, Uney JB, Wraith D, Scolding NJ (2008) Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. Neurosci. Lett 448:71–73.

Gordon D, Pavlovska G, Uney JB, Wraith DC, Scolding NJ (2010) Human mesenchymal stem cells infiltrate the spinal cord, reduce demyelination, and

localize to white matter lesions in experimental autoimmune encephalomyelitis. J. Neuropathol. Exp. Neurol 69:1087–1095.

Graham V, Khudyakov J, Ellis P, Pevny L (2003) SOX2 functions to maintain neural progenitor identity. Neuron 39:749–765.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL (1996) Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J. Neurosci 16:1091–1100.

Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc. Natl. Acad. Sci. U.S.A 99:2199–2204.

Hsieh J, Aimone JB, Kaspar BK, Kuwabara T, Nakashima K, Gage FH (2004) IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. J. Cell Biol 164:111–122.

Hu Q, Zhang L, Wen J, Wang S, Li M, Feng R, Yang X, Li L (2010) The EGF receptor-sox2-EGF receptor feedback loop positively regulates the self-renewal of neural precursor cells. Stem Cells 28:279–286.

Izrael M, Zhang P, Kaufman R, Shinder V, Ella R, Amit M, Itskovitz-Eldor J, Chebath J, Revel M (2007) Human oligodendrocytes derived from embryonic stem cells: Effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. Mol. Cell. Neurosci 34:310–323.

Jayakumar AR, Norenberg MD (2010) The Na-K-Cl Co-transporter in astrocyte swelling. Metab Brain Dis 25:31–38.

Johansson CB, Svensson M, Wallstedt L, Janson AM, Frisén J (1999) Neural stem cells in the adult human brain. Exp. Cell Res 253:733–736.

Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 10:3129–3140.

Kandel ER (2000) Michael V.L. Bennett and the cellular study of neural systems at Albert Einstein and Woods Hole. Brain Res. Brain Res. Rev 32:3–5.

Kassis I, Grigoriadis N, Gowda-Kurkalli B, Mizrachi-Kol R, Ben-Hur T, Slavin S, Abramsky O, Karussis D (2008) Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. Arch. Neurol 65:753–761.

Kaur C, Hao AJ, Wu CH, Ling EA (2001) Origin of microglia. Microsc. Res. Tech 54:2–9.

Kemp K, Gordon D, Wraith DC, Mallam E, Hartfield E, Uney J, Wilkins A, Scolding N (2011) Fusion between human mesenchymal stem cells and rodent cerebellar Purkinje cells. Neuropathol. Appl. Neurobiol 37:166–178.

Kempermann G, Gage FH (2002) Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. Brain Res. Dev. Brain Res 134:1–12.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447–452.

Knapp PE, Skoff RP, Sprinkle TJ Differential expression of galactocerebroside, myelin basic protein, and 2',3'-cyclic nucleotide 3'-phosphohydrolase during development of oligodendrocytes in vitro. J. Neurosci. Res 21:249–259.

Kokaia Z, Lindvall O (2003) Neurogenesis after ischaemic brain insults. Curr. Opin. Neurobiol 13:127–132.

Kopen GC, Prockop DJ, Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc. Natl. Acad. Sci. U.S.A 96:10711–10716.

Krabbe C, Zimmer J, Meyer M Neural transdifferentiation of mesenchymal stem cells--a critical review. APMIS 113:831–844.

Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377.

Kukekov VG, Laywell ED, Suslov O, Davies K, Scheffler B, Thomas LB, O'Brien TF, Kusakabe M, Steindler DA (1999) Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp. Neurol 156:333–344.

Lanza C, Morando S, Voci A, Canesi L, Principato MC, Serpero LD, Mancardi G, Uccelli A, Vergani L (2009) Neuroprotective mesenchymal stem cells are endowed with a potent antioxidant effect in vivo. J. Neurochem 110:1674–1684.

Lecoeur H (2002) Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp. Cell Res 277:1–14.

Lee PH, Kim JW, Bang OY, Ahn YH, Joo IS, Huh K (2008) Autologous mesenchymal stem cell therapy delays the progression of neurological deficits in patients with multiple system atrophy. Clin. Pharmacol. Ther 83:723–730.

Li Y, Chen J, Wang L, Zhang L, Lu M, Chopp M (2001) Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Neurosci. Lett 316:67–70.

Li Y, Chen J, Zhang CL, Wang L, Lu D, Katakowski M, Gao Q, Shen LH, Zhang J, Lu M, Chopp M (2005) Gliosis and brain remodeling after treatment of stroke in rats with marrow stromal cells. Glia 49:407–417.

Li Y, Chopp M, Chen J, Wang L, Gautam SC, Xu YX, Zhang Z (2000) Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. J. Cereb. Blood Flow Metab 20:1311–1319.

Liu Y, Rao MS (2004) Glial progenitors in the CNS and possible lineage relationships among them. Biol. Cell 96:279–290.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

Lobjois V, Bel-Vialar S, Trousse F, Pituello F (2008) Forcing neural progenitor cells to cycle is insufficient to alter cell-fate decision and timing of neuronal differentiation in the spinal cord. Neural Dev 3:4.

Lu P, Blesch A, Tuszynski MH (2004) Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J. Neurosci. Res 77:174–191.

Lu P, Jones LL, Tuszynski MH (2005) BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Exp. Neurol 191:344–360.

Mahmood A, Lu D, Wang L, Li Y, Lu M, Chopp M (2001) Treatment of traumatic brain injury in female rats with intravenous administration of bone marrow stromal cells. Neurosurgery 49:1196-203; discussion 1203-4.

Maiese K, Chong ZZ, Shang YC (2008) OutFOXOing disease and disability: the therapeutic potential of targeting FoxO proteins. Trends Mol Med 14:219–227.

Markakis EA, Palmer TD, Randolph-Moore L, Rakic P, Gage FH (2004) Novel neuronal phenotypes from neural progenitor cells. J. Neurosci 24:2886–2897.

Mayer M, Bhakoo K, Noble M (1994) Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. Development 120:143–153.

Miller RH (2002) Regulation of oligodendrocyte development in the vertebrate CNS. Prog. Neurobiol 67:451–467.

Ming G, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu. Rev. Neurosci 28:223–250.

Minguell JJ, Erices A, Conget P (2001) Mesenchymal stem cells. Exp. Biol. Med. (Maywood) 226:507–520.

Molofsky AV, Pardal R, Iwashita T, Park I, Clarke MF, Morrison SJ (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425:962–967.

Momma S, Johansson CB, Frisén J (2000) Get to know your stem cells. Curr. Opin. Neurobiol 10:45–49.

Mondal D, Pradhan L, LaRussa VF (2004) Signal transduction pathways involved in the lineage-differentiation of NSCs: can the knowledge gained from blood be used in the brain? Cancer Invest 22:925–943.

Moore KA, Lemischka IR (2006) Stem cells and their niches. Science 311:1880–1885.

Morrison SJ, Shah NM, Anderson DJ (1997) Regulatory mechanisms in stem cell biology. Cell 88:287–298.

Muñoz-Elías G, Woodbury D, Black IB (2003) Marrow stromal cells, mitosis, and neuronal differentiation: stem cell and precursor functions. Stem Cells 21:437–448.

Napoli I, Neumann H (2009) Microglial clearance function in health and disease. Neuroscience 158:1030–1038.

Navascués J, Calvente R, Marín-Teva JL, Cuadros MA (2000) Entry, dispersion and differentiation of microglia in the developing central nervous system. An. Acad. Bras. Cienc 72:91–102.

Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J. Neurosci. Res 77:192–204.

Neuhuber B, Timothy Himes B, Shumsky JS, Gallo G, Fischer I (2005) Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. Brain Res 1035:73–85.

Olstorn H, Varghese M, Murrell W, Moe MC, Langmoen IA (2011) Predifferentiated brain-derived adult human progenitor cells migrate toward ischemia after transplantation to the adult rat brain. Neurosurgery 68:213-22; discussion 222.

Ostenfeld T, Svendsen CN (2003) Recent advances in stem cell neurobiology. Adv Tech Stand Neurosurg 28:3–89.

Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, Straube A (2003) Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. Cell Transplant 12:839–848.

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J. Neurosci 19:8487–8497.

Palmer TD, Ray J, Gage FH (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol. Cell. Neurosci 6:474–486.

Palmer TD, Takahashi J, Gage FH (1997) The adult rat hippocampus contains primordial neural stem cells. Mol. Cell. Neurosci 8:389–404.

Parr AM, Kulbatski I, Wang X, Keating A, Tator CH (2008) Fate of transplanted adult neural stem/progenitor cells and bone marrow-derived mesenchymal stromal cells in the injured adult rat spinal cord and impact on functional recovery. Surg Neurol 70:600-7; discussion 607.

Pedraza CE, Monk R, Lei J, Hao Q, Macklin WB (2008) Production, characterization, and efficient transfection of highly pure oligodendrocyte precursor cultures from mouse embryonic neural progenitors. Glia 56:1339–1352.

Pontikoglou C, Delorme B, Charbord P (2008) Human bone marrow native mesenchymal stem cells. Regen Med 3:731–741.

Popov SG, Villasmil R, Bernardi J, Grene E, Cardwell J, Wu A, Alibek D, Bailey C, Alibek K (2002) Lethal toxin of Bacillus anthracis causes apoptosis of macrophages. Biochem. Biophys. Res. Commun 293:349–355.

Qu Q, Shi Y (2009) Neural stem cells in the developing and adult brains. J. Cell. Physiol 221:5–9.

Rao MS (2005) Neural development and stem cells. Totowa, N.J: Humana Press.

Ray J, Gage FH (2006) Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. Mol. Cell. Neurosci 31:560–573.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Rismanchi N, Floyd CL, Berman RF, Lyeth BG (2003) Cell death and long-term maintenance of neuron-like state after differentiation of rat bone marrow stromal cells: a comparison of protocols. Brain Res 991:46–55.

Rivera FJ, Couillard-Despres S, Pedre X, Ploetz S, Caioni M, Lois C, Bogdahn U, Aigner L (2006) Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. Stem Cells 24:2209–2219.

Rivera FJ, Kandasamy M, Couillard-Despres S, Caioni M, Sanchez R, Huber C, Weidner N, Bogdahn U, Aigner L (2008) Oligodendrogenesis of adult neural progenitors: differential effects of ciliary neurotrophic factor and mesenchymal stem cell derived factors. J. Neurochem 107:832–843.

Rivera FJ, Steffenhagen C, Kremer D, Kandasamy M, Sandner B, Couillard-Despres S, Weidner N, Küry P, Aigner L (2010) Deciphering the oligodendrogenic program

of neural progenitors: cell intrinsic and extrinsic regulators. Stem Cells Dev 19:595–606.

Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. Neuron 39:13–25.

Sakimoto I, Ohta K, Yamazaki T, Ohtani S, Sahara H, Sugawara F, Sakaguchi K, Miura M (2006) Alpha-sulfoquinovosylmonoacylglycerol is a novel potent radiosensitizer targeting tumor angiogenesis. Cancer Res 66:2287–2295.

Samanta J, Kessler JA (2004) Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development 131:4131–4142.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp. Neurol 164:247–256.

Sansom SN, Griffiths DS, Faedo A, Kleinjan D, Ruan Y, Smith J, van Heyningen V, Rubenstein JL, Livesey FJ (2009) The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:e1000511.

Santa María L, Rojas CV, Minguell JJ (2004) Signals from damaged but not undamaged skeletal muscle induce myogenic differentiation of rat bone-marrow-derived mesenchymal stem cells. Experimental Cell Research 300:418–426.

Schousboe A, Sickmann HM, Walls AB, Bak LK, Waagepetersen HS (2010) Functional importance of the astrocytic glycogen-shunt and glycolysis for maintenance of an intact intra/extracellular glutamate gradient. Neurotox Res 18:94–99.

Seaberg RM, van der Kooy D (2002) Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. J. Neurosci 22:1784–1793.

Seaberg RM, van der Kooy D (2003) Stem and progenitor cells: the premature desertion of rigorous definitions. Trends Neurosci 26:125–131.

Seri B, García-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J. Neurosci 21:7153–7160.

Shen Q, Wang Y, Dimos JT, Fasano CA, Phoenix TN, Lemischka IR, Ivanova NB, Stifani S, Morrisey EE, Temple S (2006) The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. Nat. Neurosci 9:743–751.

Siegrist SE, Haque NS, Chen C, Hay BA, Hariharan IK (2010) Inactivation of both Foxo and reaper promotes long-term adult neurogenesis in Drosophila. Curr. Biol 20:643–648.

Sim FJ, Zhao C, Penderis J, Franklin RJM (2002) The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. J. Neurosci 22:2451–2459.

Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat. Rev. Genet 8:263–271.

Squire LR (2008) Fundamental neuroscience. Amsterdam ;, Boston: Elsevier / Academic Press.

Stanfield BB, Trice JE (1988) Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. Exp Brain Res 72:399–406.

Steffenhagen C, Kraus S, Dechant F, Kandasamy M, Lehner B, Poehler A, Furtner T, Siebzehnrubl FA, Couillard-Despres S, Strauss O, Aigner L, Rivera FJ (2011) Identity, Fate and Potential of Cells Grown as Neurospheres: Species Matters. Stem cell reviews.

Taupin P, Gage FH (2002) Adult neurogenesis and neural stem cells of the central nervous system in mammals. J. Neurosci. Res 69:745–749.

Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY (2002) Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc. Natl. Acad. Sci. U.S.A 99:3024–3029.

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545.

Vassilopoulos G, Wang P, Russell DW (2003) Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904.

Vazey EM, Connor B (2009) In vitro priming to direct neuronal fate in adult neural progenitor cells. Exp. Neurol 216:520–524.

Wachs F, Couillard-Despres S, Engelhardt M, Wilhelm D, Ploetz S, Vroemen M, Kaesbauer J, Uyanik G, Klucken J, Karl C, Tebbing J, Svendsen C, Weidner N, Kuhn H, Winkler J, Aigner L (2003) High efficacy of clonal growth and expansion of adult neural stem cells. Lab. Invest 83:949–962.

Wang S, Sdrulla A, Johnson JE, Yokota Y, Barres BA (2001) A role for the helix-loop-helix protein Id2 in the control of oligodendrocyte development. Neuron 29:603–614.

Watanabe M, Sakurai Y, Ichinose T, Aikawa Y, Kotani M, Itoh K (2006) Monoclonal antibody Rip specifically recognizes 2',3'-cyclic nucleotide 3'-phosphodiesterase in oligodendrocytes. J. Neurosci. Res 84:525–533.

Weimann JM, Charlton CA, Brazelton TR, Hackman RC, Blau HM (2003) Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. Proc. Natl. Acad. Sci. U.S.A 100:2088–2093.

Weimann JM, Johansson CB, Trejo A, Blau HM (2003) Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. Nat. Cell Biol 5:959–966.

Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100:157–168.

Welsch U, Sobotta J (2006) Lehrbuch Histologie. Zytologie, Histologie, mikroskopische Anatomie ; mit 21 Tabellen. München ;, Jena: Elsevier, Urban und Fischer.

Williams A, Piaton G, Aigrot M, Belhadi A, Théaudin M, Petermann F, Thomas J, Zalc B, Lubetzki C (2007) Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis? Brain 130:2554–2565.

Woodbury D, Reynolds K, Black IB (2002) Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res 69:908–917.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res 61:364–370.

Ying Q, Nichols J, Evans EP, Smith AG (2002) Changing potency by spontaneous fusion. Nature 416:545–548.

Zhang J, Brodie C, Li Y, Zheng X, Roberts C, Lu M, Gao Q, Borneman J, Savant-Bhonsale S, Elias SB, Chopp M (2009) Bone marrow stromal cell therapy reduces proNGF and p75 expression in mice with experimental autoimmune encephalomyelitis. J. Neurol. Sci 279:30–38.

Zhang J, Li Y, Chen J, Cui Y, Lu M, Elias SB, Mitchell JB, Hammill L, Vanguri P, Chopp M (2005) Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. Exp. Neurol 195:16–26.

Zhang J, Li Y, Chen J, Yang M, Katakowski M, Lu M, Chopp M (2004) Expression of insulin-like growth factor 1 and receptor in ischemic rats treated with human marrow stromal cells. Brain Res 1030:19–27.

Zhang J, Li Y, Lu M, Cui Y, Chen J, Noffsinger L, Elias SB, Chopp M (2006) Bone marrow stromal cells reduce axonal loss in experimental autoimmune encephalomyelitis mice. J. Neurosci. Res 84:587–595.

Zhou Q, Anderson DJ (2002) The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109:61–73.

Publication in Stem cells and development

Mesenchymal Stem Cells Prime Proliferating Adult Neural Progenitors Towards an Oligodendrocyte Fate

Carolin Steffenhagen[1*], Franz-Xaver Dechant[1,2*], Eleni Oberbauer[1], Tanja Furtner[1], Norbert Weidner[3], Patrick Küry[4], Ludwig Aigner[1#], Francisco J. Rivera[1,2#]

[1]Institute of Molecular Regenerative Medicine, Paracelsus Medical University Salzburg, Strubergasse 21, 5020 Salzburg, Austria. Phone: +43 662 44 20021280, Fax: +43 662 44 20021209.

[2]Department of Neurology, University of Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg, Germany. Phone: +49 941 9448950, Fax: +49 941 9448951.

[3]Clinic of Paraplegiology, University of Heidelberg, Schlierbacher Landstrasse 200a, 69118 Heidelberg, Germany. Phone +49-6221-966322, Fax: +49 6221 966345.

[4]Department of Neurology, Heinrich-Heine-University, Moorenstrasse 5, 40225 Düsseldorf, Germany. Phone +49 211 8117822, Fax: +49 211 8118411.

* These authors equally contributed to this study

Shared senior authorship

Email addresses:

Carolin Steffenhagen: c.steffenhagen@pmu.ac.at

Franz-Xaver Dechant: franzdechant@web.de

Eleni Oberbauer: eleni.oberbauer@pmu.ac.at

Tanja Furtner: tanja.furtner@pmu.ac.at

Norbert Weidner: norbert.weidner@med.uni-heidelberg.de

Patrick Küry: kuery@uni-duesseldorf.de

Ludwig Aigner: ludwig.aigner@pmu.ac.at

Francisco J. Rivera: francisco.rivera@pmu.ac.at

Running Title: MSCs prime neural progenitors to oligodendrocytes

Correspondence:

Ludwig Aigner, PhD, Institute of Molecular Regenerative Medicine, Paracelsus Medical University Salzburg, Strubergasse 21, 5020 Salzburg, Austria, Phone: +43 662 44 20021280, Fax: +43 662 44 20021209, email: ludwig.aigner@pmu.ac.at

Abstract

Oligodendrogenesis encompasses lineage specification of neural progenitors (NPCs) and differentiation into oligodendrocytes, which ultimately culminates in the myelination of central nervous system axons. Each individual process must be tightly regulated by extracellular and cell-intrinsic mechanisms, whose identities are barely known. We had previously demonstrated that soluble factors derived from rat mesenchymal stem cells (MSCs) induce oligodendrogenesis in differentiating adult NPCs under differentiation conditions. However, since lineage specification predominantly occurs in proliferating progenitors and not necessarily during early differentiation, we investigated if soluble factors derived from MSCs are able to prime NPCs to the oligodendroglial fate already under proliferation conditions. Therefore, we analyzed the effects of a three weeks stimulation of adult NPCs under proliferation conditions with conditioned media derived from MSCs (MSC-CM) in terms of cell morphology, proliferation, cell-specific marker expression profile, response to growth factor withdrawal, cell-lineage restriction, and expression of glial fate determinants. While MSC-CM did not

affect the proliferation rate of NPCs, it boosted the formation of CNPase- as well as MBP-expressing oligodendrocytes after growth factor withdrawal, even when cells were exposed to an astrogenic milieu. Moreover, it reinforced the proper development of oligodendrocytes, since it ensured a sustained expression of the functional marker CNPase. Finally, in proliferating NPCs, the presence of MSC-CM reduced the anti-oligodendrogenic determinant Id2, thus increasing the relative proportion of the pro-oligodendrogenic factor Olig2 expression. In summary, MSCs prime proliferating progenitors and, thus, reinforce cell fate choice and accelerate differentiation towards the oligodendrocyte lineage. The present findings underscore the potential use of MSCs in cell therapies for remyelination such as in multiple sclerosis and spinal cord injury. Moreover, they urge the identification of the oligodendrogenic activity(ies) derived from MSCs in order to develop novel molecular therapies for demyelinating diseases.

Introduction

Oligodendrocytes are one of the three main neuroectodermal cell types within the central nervous system (CNS). Their main function is to form myelin that wraps axons to facilitate saltatory electric conductance [1]. Demyelinating diseases, such as multiple sclerosis (MS), are characterized by the loss of oligodendrocytes resulting in severe neurological symptoms such as hemiparesis or visual impairment. Enhancing oligodendrogenesis and promoting remyelination might be an attractive approach to counteract this situation. The feasibility of this approach is provided by the fact that oligodendrogenesis can occur during adulthood. Oligodendrocyte progenitors cells (OPCs) are widespread throughout the CNS in white and grey matter, representing 5 to 8% of total glial cells [2,3] and constitute the major cellular source of remyelinating cells [4]. Upon demyelination, endogenous OPCs start to proliferate and the expression of oligodendrogenic genes is induced. After OPCs activation, cells are recruited towards the lesion site where they differentiate and maturate into myelinating oligodendrocytes [4,5]. However, OPCs are not unique in the ability to remyelinate, since subventricular zone (SVZ) derived neural stem cells (NSCs), beside their neurogenic potential, represent a source for new oligodendrocytes [6-9]. Under normal physiological conditions as well as in response to demyelinating insults, SVZ derived NSCs migrate into the corpus callosum, the striatum and to the fimbria fornix where they give rise to myelinating oligodendrocytes [6-8,10]. In addition to this, cells residing in the subcallosum zone (SCZ) also enter the corpus collosum and generate oligodendrocytes [11]. Therefore, the adult CNS is equipped with different cellular sources for remyelination and regenerative mechanisms in response to demyelination.

Oligodendrogenesis is a hierarchically structured process that involves i) specification of proliferating neural stem / progenitor cells to the oligodendroglial lineage via glial and subsequently oligodendroglial progenitors, ii) migration of progenitors and differentiation into oligodendrocytes, and iii) myelination of axons [12-14] (Fig. 1A). This process involves the sequential generation of several cell types that display distinct proliferation and differentiation properties. Due to the cell-specific marker expression profile, each cell type can be monitored and identified *in vivo* and *in vitro* (Fig. 1A). Each individual step in oligodendrogenesis is tightly regulated by extracellular and cell-intrinsic mechanisms. For example, platelet derived growth factor (PDGF) and sonic hedgehog (Shh) are potent activators of oligodendrogenesis [15-18], while bone morphogenetic proteins (BMPs) inhibit the generation of oligodendrocytes. BMPs induce the expression of the inhibitors of differentiation 2 and 4 (Id2 and Id4), which sequester the pro-oligodendrogenic transcription factors Olig1 and Olig2 in the cytoplasm avoiding their entrance into the nucleus [19]. Therefore, Olig1/2 and Id2/4 proteins are glial fate determinants and the balance between their expression level plays a crucial role for the astrocyte / oligodendrocyte decision.

Recently, we and others had shown that conditioned medium derived from mesenchymal stem cells (MSCs) promote expression of myelin basic protein (MBP) and oligodendrogenesis in differentiating NPCs [20-22]. Moreover, we demonstrated that MSCs promote oligodendroglial differentiation of NPCs that were co-transplanted into a hippocampal slice [23]. Thus, factors derived from MSCs strongly promote oligodendrogenesis of differentiating cells. It is, however, unclear, if MSC-CM targets progenitors and specifies them towards the oligodendroglial fate also under proliferation conditions. Obviously, this is of huge relevance, since proliferating progenitors commonly surround CNS lesions in high numbers, but often fail to remyelinate sparse axons [24,25]. Moreover, this situation becomes more evident and

relevant in aged subjects [26-28]. While the deficiency to remyelinate is partly derived from the failure to differentiate into oligodendroglia [24,26-28], a lack of sufficient oligodendroglial commitment in proliferating progenitors might contribute to this deficiency. Here, we test the hypothesis that MSC derived soluble factors are able to predispose or prime proliferating NPCs towards an oligodendroglial fate.

Materials and Methods

Animal subjects

Adult female Fischer 344 rats (Charles River Deutschland GmbH, Germany) were used as donors for the NPCs and MSCs cultures. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. Animals had *ad libitum* access to food and water throughout the study.

Rat NPCs cultures and neurospheres formation

NPCs derived from the adult hippocampus were generated as described [20,29]. Briefly, 2 to 4 month-old rats (Charles River Deutschland GmbH, Germany) were decapitated. Hippocampi were aseptically removed, transferred to 4°C DPBS (PAN, Germany) with 4.5g/L glucose (Merck, Germany) (DPBS/glu), washed once, transferred to petri dishes and dissociated mechanically. The cell suspension was washed in DPBS/glu and resuspended in PPD-solution containing 0.01% Papain (Worthington Biochemicals, England), 0.1% dispase II (Boehringer, Germany), 0.01% DNase I (Worthington Biochemicals, England) and 12.4 mM MgSO4 in HBSS (PAN, Germany) without Mg^{++}/Ca^{++} (PAA, Germany) and digested for 30 to 40 min at 37°C. The cell suspension was triturated every 10 min. Dissociated cells were

collected and resuspended in Neurobasal (NB) medium containing B27 (Gibco BRL, Germany), 2mM L-glutamine and 100U/mL penicillin/ 100 µg/ml streptomycin and washed three times. Finally, the single cell suspension was resuspended in NB medium (Gibco BRL, Germany) supplemented with B27 (Gibco BRL, Germany) (NB/B27), 2 mM L-glutamine (PAN, Germany), 100U/mL penicillin/ 100 µg/ml streptomycin (PAN, Germany), 2 µg/ml heparin (Sigma, Germany), human recombinant 20 ng/ml FGF-2 (R&D Systems GmbH, Germany) and human recombinant 20 ng/ml EGF (R&D Systems GmbH, Germany). Cultures were maintained at 37°C in an incubator with 5% CO_2. Half of the medium was changed every 3 to 4 days. After 1 to 2 weeks NPCs formed neurospheres and were passaged. Thereafter, neurospheres were passaged once a week and were grown for 2 to 3 weeks to reach passage 2. Neurospheres cultures from passage number 2 to 6 were used throughout this study.

Rat MSCs cultures and conditioned medium preparation (MSC-CM)

MSCs were prepared as described previously [20]. Briefly, bone marrow plugs were harvested from femurs and tibias of 2-4 month-old female Fisher-344 rats (Charles River Deutschland GmbH, Germany). Plugs were mechanically dissociated in αMEM (Gibco Invitrogen, Germany) and recovered by centrifugation. Cell pellets were resuspended in αMEM-10% FBS and seeded at $1x10^6$ cells/cm^2. After 3 days, media was changed and non-adherent cells were removed. Adherent cells were incubated in fresh αMEM-10% FBS until a confluent layer of cells was achieved. Cells were trypsinized using 0.25% Trypsin (Gibco Invitrogen, Germany) and seeded in αMEM-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 further cultured for experiments or frozen for later use. As

demonstrated in our previous work, this cell culture preparation is highly enriched in multipotent MSCs with virtually no hematopoietic contamination [20].

Mesenchymal stem cell conditioned medium (MSC-CM) was prepared similar as described [20] with the exception of the media used. MSCs were plated at 12,000 cells/cm^2 and incubated in normal NPCs proliferation medium (NB medium supplemented with B27 (NB/B27), 2 mM L-glutamine, 100U/mL penicillin/ 100 µg/ml streptomycin, 2 µg/ml heparin, human recombinant 20 ng/ml FGF-2 and human recombinant EGF 20 ng/ml). After 3 days, the conditioned medium was collected and filtered using a 0.22 µm-pore filter. In some of the experiments, the conditioned medium was replenished with EGF and FGF-2 (20 ng/ml each) to exclude the possibility that MSCs might have consumed the growth factors.

NPCs stimulation with MSC-CM and cell number analysis

5x10^4 NPCs/ml were incubated up to 3 weeks either in normal NPCs proliferating medium (control) or in complete MSC-CM (MSC-CM). Alternatively, cells were incubated in a half/half mixture between normal NPCs proliferation media and MSC-CM (50% MSC-CM) or in MSC-CM comprising 20 ng/ml FGF-2 and 20 ng/ml EGF (MSC-CM + GF). Media was changed after 3 to 4 days.

Neurospheres proliferation was explored by determining the cell number for control condition, 50% MSC-CM, 100% MSC-CM and MSC-CM + GF through Trypan blue (Sigma-Aldrich, Germany) exclusion and MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Germany) as well as by measuring the number of neurospheres and the mean neurosphere diameter.

For Trypan blue exclusion $5x10^4$ cells/ml were seeded in the above mentioned media. After 7, 14 and 21 days, cell number was determined by Trypan blue exclusion. The neurospheres and the cells that were adherent after MSC-CM treatment were detached and dissociated with Accutase and treated with Trypan blue. Following, the number of living cells was determined using a light microscope (Zeiss, Germany).

For the MTS assay, untreated cells and cells that were treated for one week with 50% MSC-CM, 100% MSC-CM or MSC-CM + GF were seeded in the respective media into 96-well plates at a density of $2x10^4$ cells/cm^2. The assay was performed according to the manufacturer's instructions. On day 0, 2, 4 and 7 the optical density was measured at 490nm in a microplate reader (Berthold Technologies, Austria) after 3 hours of incubation with the MTS solution.

Similar to the MTS assay cells in normal proliferation media and cells that were treated for one week with 50% MSC-CM, 100% MSC-CM or MSC-CM + GF were used to determine the neurospheres number and diameter. Cells were seeded at a density of $1,5x10^4$ cells/cm^2 and incubated in the respective media for 2, 4 or 7 days. The number of neurospheres was analyzed by Trypan blue exclusion under a light microscope. The neurospheres diameter was determined using Olympus IX81 inverted microscope equipped with Hamamatsu digital camera and Volocity 5.4.1 software (PerkinElmer, Germany).

Expression profile analysis of cell-lineage-specific markers

NPCs were incubated under normal proliferating medium (control), 50% MSC-CM, 100% MSC-CM, and MSC-CM replenished with growth factors (MSC-CM + GF). After 3

weeks, marker expression profile was determined. For some experiments, RNA was isolated directly from the neurospheres after the 3 weeks treatment period and processed for qRT-PCR. Alternatively, the floating neurospheres as well as the cells that got adherent under the MSC-CM treatment were dissociated with Accutase (Innovative Cell Technologies Inc., distributed by PAA) and then plated on 100 µg/ml poly-L-ornithine (Sigma-Aldrich, Germany) and 5 µg/ml laminin-coated (Sigma-Aldrich, Germany) glass coverslips ($2.5x10^4$ cells/cm^2) for subsequent immunofluorescence stainings or seeded in 100 µg/ml poly-L-ornithine and 5 µg/ml laminin-coated plates ($2x10^4$ cells/cm^2) for subsequent RNA isolation. The dissociated cells were incubated in DMEM Knockout-20% Serum Replacement supplement (SR) (Gibco Invitrogen, Germany) for 12 hours. This serum-free media has been used for embryonic stem cell maintenance and does not induce differentiation [30-32]. Under these conditions dissociated NPCs attach to the plate in the absence of serum. To analyze the expression of cell-lineage-specific markers, cells were either fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence or RNA was isolated from the cells and used for qRT-PCR.

Growth factor withdrawal response

NPCs were incubated under normal proliferation medium (control), 50% MSC-CM, 100% MSC-CM, and MSC-CM replenished with growth factors (MSC-CM + GF). After 3 weeks, the response to growth factor withdrawal was determined. First, floating neurospheres as well as the cells that were adherent after MSC-CM treatment were detached and dissociated with Accutase (Innovative Cell Technologies Inc., distributed by PAA). Then, the dissociated cells were plated on 100 µg/ml poly-L-ornithine (Sigma-Aldrich, Germany) and 5 µg/ml laminin-coated (Sigma-Aldrich, Germany) glass coverslips ($2.5x10^4$ cells/cm^2) for subsequent immunofluorescence stainings or directly in 100 µg/ml poly-L-ornithine and 5 µg/ml

laminin-coated plates ($2.5x10^4$ cells/cm^2) for subsequent RNA isolation. The cells were incubated in DMEM Knockout-20% Serum Replacement supplement (SR) (Gibco Invitrogen, Germany) for 3 or 7 days. Cells were then fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence or, alternatively, RNA was isolated from the cells and used for qRT-PCR.

Astrogenic potential and cell-lineage restriction analysis

NPCs were incubated with normal proliferation medium (control), 50% MSC-CM and 100% MSC-CM. After 3 weeks, the astrogenic potential was determined. Floating neurospheres as well as the cells that were adherent after MSC-CM treatment were detached and dissociated with Accutase (Innovative Cell Technologies Inc., distributed by PAA) and plated on 100 µg/ml poly-L-ornithine (Sigma-Aldrich, Germany) and 5 µg/ml laminin-coated (Sigma-Aldrich, Germany) glass coverslips at a density of $2.5x10^4$ cells/cm^2 in αMEM (Gibco Invitrogen, Germany) and 10% FBS (Lonza, Germany) was added as an astrogenic stimulus. After 7 days, cells were fixed for 30 minutes with 4% paraformaldehyde and processed for immunofluorescence to detect the expression of the different cell-lineage-specific markers.

Immunofluorescence

Fixed cells were washed in TBS (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5), then blocked with solution composed of TBS; 0.1% Triton-X100 (only for intracellular antigens); 1% bovine serum albumin (BSA) and 0.2% Teleostean gelatin (Sigma, Germany) (fish gelatin buffer, FGB). The same solution was used during the incubations with antibodies. Primary antibodies were applied overnight at 4°C. Fluorochrome-conjugated species-specific secon-

dary antibodies were used for immunodetection. The following antibodies and final dilutions were used. Primary antibodies: rabbit anti-GFAP 1:1000 (Dako, Denmark); mouse anti-ratNestin 1:500 (Pharmingen, Germany); goat anti-Sox2 1:1000 (Santa Cruz, Germany); IgM mouse anti-O4 1:100 (Chemicon, USA); rabbit anti-platelet-derived growth factor receptor alpha (PDGFRα) 1:100 (Santa Cruz, Heidelberg, Germany); IgM mouse anti-A2B5 1:200 (Chemicon, UK); mouse anti-Map 2a+2b 1:250 (Sigma, Germany); rabbit anti-doublecortin (DCX) 1:500 (NEB, Frankfurt, Germany); mouse anti-2', 3'-cyclic-nucleotide-3'-phosphodieesterase (CNPase) 1:200 (Millipore, USA); rabbit anti-NG2 1:200 (Millipore, USA); rabbit anti-Galactocerebroside (GalC) 1:200 (Millipore, USA); mouse anti-RIP 1:200 (Millipore, USA); mouse anti-Myelin Basic Protein (MBP) 1:750 (SMI-94, Sternberger Monoclonals Incorporated, U.S.A.). Secondary antibodies: donkey anti-mouse, rabbit conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA), rhodamine X 1:1000 (Dianova, Hamburg, Germany) and donkey anti-goat conjugated with Alexa Fluor 568 1:1000 (Invitrogen, Germany). In cases of detergent-sensitive antigens (i.e. O4, A2B5, GalC and NG2), Triton X-100 was omitted from FGB buffer. Nuclear counterstaining was performed with 4´, 6´-diamidino-2-phenylindole dihydrochloride hydrate at 0.25 µg/µl (DAPI; Sigma, Germany). Specimens were mounted on microscope slides using in Prolong Antifade kit (Molecular Probes, U.S.A.). Epifluorescence observation and photo-documentation were realized using a Leica microscope (Leica Mikroskopie und Systeme GmbH, Germany) equipped with a Spot™ digital camera (Diagnostic Instrument Inc, U.S.A.) or an Olympus IX81 inverted microscope equipped with Hamamatsu digital camera and Volocity 5.4.1 software (PerkinElmer, Germany). For each culture condition, 10 randomly selected observation fields, containing in total 500-1,000 cells, were photographed for cell fate analysis. Expression frequency of selected cell type markers was determined for every condition in three independent experiments.

Quantitative PCR

RNA extraction from NPCs was performed using RNeasy Mini Kit (Qiagen, Germany) and cDNA was synthetized using Promega reverse transcription Kit (Promega, Germany). Expression analysis was performed by TaqMan gene expression assays kits (Applied Biosystems, USA) for the following rat genes: Olig1, Olig2, Id2, NG2, Nestin, PDGFRα, CNPase and MBP. Probes and primers were provided by the manufacturer (Applied Biosystems, USA). Rat glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene. The following temperature profile was used: activation of polymerase 95°C, 10 min; 40 cycles of denaturing 95°C, 15 s, and annealing/extension 60°C, 60 s. Data was obtained with a Rotor-Gene 6000 R Corbett Research (geneXpress, Austria) and analyzed by delta delta Ct method [33]. Gene expression under control conditions was used as a calibrator gene.

Statistical Analysis

Data are presented as means ± SD and statistical analysis was performed using PRISM4 (GraphPad, San Diego, CA, USA). P values of $< 0,05$ were considered to be significant acquired by parametric one-way ANOVA-Tukey post hoc. For time course experiments two-way ANOVA-Bonferroni post hoc was conducted. All experiments were performed at least in triplicate in three independent experiments.

Results

Here, we used a neurosphere culture model to analyze the effects of a 3 weeks mesenchymal stem cell derived conditioned medium (MSC-CM) treatment of NPCs under prolifera-

tion conditions with the hypothesis that the MSC-CM might prime the NPCs towards an oligodendroglial fate. To test whether the NPCs used in the present study are multipotent self-renewing progenitors, we grew primary and secondary neurospheres under stringent clonal condition (one cell per well) and analyzed for neuronal, astroglial and oligodendroglial differentiation potential of the cells (see supplementary material). Even though the majority of neurosphere derived cells expressed the oligodendroglial progenitor marker O4, as demonstrated directly after seeding of the cells, approximately half of the population was positive for the neural stem cell markers Sox2 and Nestin. Moreover, the neurosphere derived cells generated primary and secondary spheres under clonal conditions and differentiated into DCX expressing neurons, GFAP positive astrocytes and MBP positive oligodendrocytes. Cell identity was analyzed directly after the 3 weeks stimulation by profiling the cell type specific marker expression. Cell intrinsic differentiation fate was analyzed after a one week period of growth factor withdrawal, and cell lineage restriction was tested by exposing the primed cells to serum, an astroglia inducing activity. Finally, we determined the expression of Olig2 and Id2 glial fate determinants on proliferating NPCs directly after the three weeks of pre-treatment (Fig. 1).

MSC-CM promotes adhesion of proliferating NPCs without interfering with cell proliferation

In order to study the effects of soluble factors derived from MSCs on proliferating NPCs, adult rat neurosphere derived single cells suspensions were treated with increasing concentrations of MSC-CM under proliferation conditions, i.e. in the presence of EGF and FGF-2 for 7, 14 or 21 days. Importantly, the basic composition of the control medium and MSC-CM were identical. One additional control condition was used for some experiments to exclude the possibility that the MSC-CM derived effects might be due to the fact that MSCs

might have consumed EGF and FGF resulting in conditioned media without growth factors. Therefore, NPCs were incubated also with MSC-CM replenished with EGF and FGF-2.

While proliferating NPCs readily re-grew neurospheres in control proliferation medium (Fig. 2A, E, I), sphere formation was reduced in 50% MSC-CM, in 100% MSC-CM and in MSC-CM + GF. Instead, cells attached to the plastic surface and formed cellular processes (Fig. 2B-D, F-H, J-L). The growth characteristics of the cultures were investigated by Trypan blue exclusion cell counting, MTS assay and by determining the number of neurospheres and the mean neurosphere diameter. Regardless of the treatment, NPC cultures expanded along the three weeks of observation with comparable kinetics by generating similar cell numbers (Fig. 2M, N) and by forming similar neurosphere numbers (Fig. 2O) with neurospheres of comparable mean diameter (Fig. 2P). The mean diameter of untreated control neurospheres was slightly, but not significantly, less compared to the MSC-CM conditions. Moreover, there were no differences in the morphology and growth characteristics of cells treated with either 100% MSC-CM or MSC-CM + GF. Taken together, this indicates that MSC-CM had no obvious influence on the growth kinetics of NPCs.

MSC-CM pre-treatment primes NPCs towards oligodendroglial differentiation under proliferation conditions

The striking effects of MSC-CM on the adherence of NPCs lead us to investigate the expression of progenitor markers in the cultures. Therefore, NPCs were cultured in EGF and FGF-2 for up to three weeks under control or MSC-CM conditions. Cells were then dissociated and seeded overnight under serum-free conditions to allow them to attach and fixed and stained for stem / progenitor markers. In consistence with our previous report [34], a subpopulation of NPCs in the cultures expressed the neural stem cell marker Nestin (between 10 to

15% Fig. 3A, B). The majority of the NPCs expressed the oligodendrocyte progenitor marker O4 (appr. 90% Fig. 3I, J) as well as the glial progenitor markers A2B5 (appr. 50% Fig. 3C, D), PDFGRα (appr. 35% Fig. 3E, F) and NG2 (appr. 30% Fig. 3G, H). The MSC-CM treatment did not affect the relative proportion of cells expressing one of these stem / progenitor markers (Fig. 3B, D, F, H, J). However, the number of RIP-positive oligodendrocytes was significantly increased after MSC-CM pre-treatment in a dose-dependant manner from approximately 0% in the control to 30% in 50% MSC-CM and to 40% in 100% MSC-CM (Fig. 3K, L). In general, fewer cells were positive for CNPase (appr. 0% in control, 2% after MSC-CM treatment; Fig. 3M, N), GalC (appr. 2% in control, 10% after MSC-CM treatment; Fig. 3O, P) or MBP (appr. 0% in control, 10% after MSC-CM treatment; Fig. 3Q, R). Finally, there were no GFAP-positive cells (Fig. 3S, T) and only very few cells that expressed the neuronal precursor marker doublecortin (DCX) in control conditions (Fig. 3U, V). The MSC-CM treatment did not affect the percentage of the DCX-positive cells (Fig. 3V) and the slight increase of GFAP-positive cells after MSC-CM treatment (Fig. 3T) was not significant. Also importantly, there was no difference in marker expression profile in cultures treated with 100% MSC-CM or MSC-CM + GF (Fig. 3L, N, P, R, T). Most of the NPCs expressed the stem / progenitor markers Nestin, A2B5, PDGFRα, NG2 and O4 indicating that the vast majority of cells in the cultures had indeed an immature character.

Next, quantitative RT-PCR was performed to determine the expression levels of neural stem cell and CNS lineage markers and to analyze the effects of the MSC-CM treatment. The three weeks MSC-CM treatment significantly enhanced the expression of CNPase- and of MBP-mRNA in the NPCs (Fig. 3W). The mRNA expression levels of the progenitor markers NG2 and PDGFRα were slightly, but not significantly, reduced, while the expression levels of Nestin and the Olig genes were not affected by the MSC-CM treatment (Fig. 3W).

These data suggest that MSC-CM primes NPCs under proliferation conditions to differentiate into oligodendrocytes. While the presence of MSC-CM induced major changes at the mRNA levels, in particular in MBP expression, the cells apparently did not yet express the oligodendrocyte phenotype at the protein level, suggesting that the oligodendrocytic phenotype at this stage is more advanced on mRNA level than on protein level.

Pre-treatment of NPCs with MSC-CM enhances growth factor withdrawal induced oligodendroglial differentiation

We recently noticed that adult rat hippocampus and SVZ derived neurospheres, although being tripotent, differentiate predominantly into oligodendrocytes after growth factor withdrawal (GFW) in serum free conditions [34].To determine whether this default differentiation pathway is affected by MSC-CM, NPCs were pre-treated with control proliferation medium or MSC-CM for three weeks, dissociated and seeded, and incubated for 7 days in serum free conditions in the absence of FGF-2 and EGF. As expected, one week after GFW, control-treated NPCs, while still expressing O4 (Fig. 4A, B), differentiated primarily into mature oligodendrocytes expressing RIP and CNPase (appr. 30% Fig. 4D, F), GalC (appr. 40% Fig. 4H), and MBP (appr. 60% Fig. 4J). Interestingly, although MSC-CM treatment did not affect the proportion of O4-expressing oligodendrocyte progenitor cells (Fig. 4A, B), it increased the percentage of RIP-, CNPase-, GalC- and MBP-expressing oligodendrocytes after GFW (Fig. 4D, F, H, J). We did not observe any MSC-CM effect on the percentage of cells that express the astrocyte marker GFAP (Fig. 4K, L). Finally, there were few cells that expressed the neuronal progenitor markers DCX (less than 4% Fig. 4M, N) and MAP2ab (less than 3% Fig. 4O, P) in response to GFW. MSC-CM pre-treatment did not affect the percentage of DCX- and MAP2ab-expressing neurons generated after GFW (Fig. 4N, P). Moreover,

regardless of the pre-treatment of proliferating NPCs, less than 10% of the cells express neural stem / progenitor markers (data not shown), indicating that in the absence of growth factors NPCs are induced to differentiate. Again, in some of the experiments, the MSC-CM was replenished with EGF and FGF-2 to exclude the possibility that MSCs might have consumed these growth factors and thereby induced the pro-oligodendroglial effects. The results, as demonstrated in Fig. 4D, F, H exclude this possibility. Interestingly, while the GFW condition demonstrated the increased oligodendroglial potential of the MSC-CM pre-treated NPCs, the mRNA levels of the pro-oligodendroglial determinants Olig1 and Olig2 and of the oligodendroglial genes CNPase and MBP were not further enhanced (Fig. 4Q). Taken together, this suggests that MSC-CM enhances the oligodendrogenic ability of proliferating NPCs resulting in an increased level of oligodendroglial differentiation in response to GFW.

MSC-CM primes proliferating NPCs: acceleration of differentiation and reinforcement of oligodendroglial commitment

Based on the fact that MSC-CM encourages proliferating NPCs towards oligodendrogenesis we asked if this effect involves accelerated oligodendrocyte differentiation / maturation and/or enhanced oligodendrocyte lineage restriction. First, to determine whether MSC-CM modulates the kinetics of oligodendrogenesis in response to GFW, we analyzed the temporal expression pattern of markers that appear sequentially along the oligodendrogenic process as well as astrocytic and neuronal markers. GFW, regardless of the pre-treatment, significantly increased the percentage of MBP-expressing mature oligodendrocytes with time (Fig. 5A). Importantly, the MSC-CM pre-treatment triggered a significant increase in the generation of MBP-expressing oligodendrocytes 3 and 7 days after GFW (Fig. 5A). The expression of GalC and of RIP after GFW significantly increased over time under all conditions analyzed (Fig. 5B, C). Moreover, the MSC-CM pre-treatment induced a dose-dependant increase in the

percentage of GalC- and of RIP positive cells (Fig. 5B, C). Although the proportion of CNPase-expressing cells transiently increased in all conditions after GFW, only MSC-CM pre-treated proliferating NPCs persistently produced such cells. This culminated in a significantly higher percentage of CNPase-positive oligodendrocytes compared to the other conditions (Fig. 5D). In addition, in spite of the fact that MSC-CM pre-treated proliferating NPCs displayed a slightly higher percentage of GFAP-expressing astrocytes compared to untreated NPCs, there was an abrupt decrease in GFAP expression after GFW independently of the pre-treatment (Fig. 5E). Finally, although minor changes in the percentage of DCX-expressing neurons were observed along time after GFW, MSC-CM pre-treatment had no significant effect on the generation of DCX-positive cells (Fig. 5F). Together, these findings indicate that MSC-CM pre-treatment of proliferating NPCs induces a faster and a more efficient differentiation into oligodendrocytes without affecting the generation of astrocytes and neurons.

Second, we analyzed the possibility that MSC-CM pre-treatment on proliferating NPCs might enhance oligodendrocyte lineage restriction. In order to test this hypothesis, MSC-CM pre-treated and non pre-treated proliferating NPCs were exposed to FBS, as serum has been described as a potent astrogenic stimulus on glial progenitors [35]. As expected, a one week stimulation with FBS predominantly induced astroglial differentiation in control-treated NPCs, since approximately 60% of the cells expressed the astrocyte marker GFAP (Fig. 6C), while only approximately 3% and 7% of the cells expressed the oligodendrocyte markers MBP and CNPase, respectively (Fig. 6A, B). In contrast, and despite the presence of an astrogenic milieu, the MSC-CM pre-treatment generated significantly more MBP- as well as CNPase-expressing oligodendrocytes (Fig. 6A, B). Moreover, this effect was at the expense of astrogenesis, since significantly less GFAP-expressing cells could be observed under these conditions (Fig. 6C). The MBP- as well as the CNPase-expressing cells in the MSC-CM pre-treated group appeared in clusters, suggesting a clonal effect. Finally, almost no DCX-

expressing cells were found under this astrogenic condition, regardless of the pre-treatment (Fig. 6D). In summary, MSC-CM pre-treatment of proliferating NPCs boosts the oligodendrocyte lineage commitment and partially counteracts the astrogenic effect of FBS.

MSC-CM increases the Olig2/Id2 ratio in proliferating NPCs during priming

Next, we reasoned that oligodendroglial priming of NPCs by MSC-CM should involve altered expression of pro- and anti-oligodendrogenic fate determinants. Here, the relative expression of Olig2 and Id2 is a key determinant for oligodendrocyte/astrocyte fate decision [19] (Fig. 1). Therefore, proliferating NPC were incubated up to 3 weeks under normal proliferation conditions (control), 50% and 100% MSC-CM, the expression level of Olig2 and Id2 were analyzed by quantitative RT-PCR, and the Olig2/Id2 ratio was determined for the different conditions. While the expression levels of Olig2 did not change within the different conditions (Fig. 7A), MSC-CM drastically reduced the expression of Id2 (Fig. 7B). In order to estimate the Olig2/Id2 ratio, the expression level of Olig2 was related to Id2 as a calibrator. MSC-CM dose-dependently increased the Olig2/Id2 ratio compared to control conditions (Fig. 7C) favoring oligodendrocyte fate decision. In conclusion, the increased Olig2/Id2 ratio in MSC-CM-treated NPCs might explain the enhanced oligodendrogenic response to GFW compared to non-treated NPCs.

Discussion

Cell progenitor priming as well as cell fate and lineage decisions presumably occur while progenitors are still proliferating [12,36-38]. For instance, different combinations of growth factors such as PDGFα, FGF-2 and Shh can convert embryonic derived proliferating

neurospheres into OPCs containing oligospheres [38,39]. Lithium chloride primes proliferating NPCs and increases the proportion of cells expressing neuronal markers at the expense of gliogenesis [37]. Moreover, FGF-2, heparin and laminin set up adult human brain derived proliferating NPCs towards a neuronal fate [36]. Here, we have shown that MSCs prime proliferating NPCs, reinforce oligodendroglial cell fate decision and accelerate the differentiation towards oligodendrocytes.

The conditioned medium derived from MSCs did not impinge on cell proliferation and neurosphere formation. However, MSC-CM primed progenitors towards oligodendrocytes as the number of RIP-positive cells as well as the mRNA expression levels of the oligodendrocyte markers CNPase and MBP were significantly enhanced after a 3 week incubation with MSC-CM under proliferation conditions. After growth factor withdrawal the MSC-CM induced elevated predisposition towards oligodengroglial differentiation at the mRNA levels consolidated into a higher percentage of RIP-, GalC-, CNPase- and MBP-positive mature oligodendrocytes. In a number of experiments, we excluded the possibility that the oligodendroglial differentiation effect of the MSC-CM might derive from a consumption of EGF or FGF-2, suggesting that the MSC-CM indeed contains a pro-oligodendroglial activity, whose identity is still under investigation. Nevertheless, the present data clearly demonstrate that the pro-oligodendroglial effect of MSC-CM is dose dependent, although, depending on the gene or protein marker analyzed, this dose-dependency might vary. This might be a consequence of minor alterations in the quality of the conditioned media resulting from slightly different features of the primary MSCs preparations. Nevertheless, in summary, regardless of the preparation, a pro-oligodendrogenic effect is always present in the MSC-CM.

The MSC-CM derived oligodendrogenic priming effect on proliferating NPCs reinforced the lineage-restriction and enables to overcome an astrogenic milieu. A surprising ob-

servation within the present work is the notion that the majority of progenitors (appr. 60%) that were generated under control conditions differentiated into MBP-expressing oligodendrocytes in serum-free conditions. This suggests that the progenitors used in the present study where already partly committed to an oligodendroglial fate, which confirms our previous work [34]. This tendency to generate oligodendrocytes might have been overlooked in many previous studies, since most of the differentiation assays are typically performed in serum-containing, and thus astrocyte-inducing, media [20,29,40].

The temporal expression pattern analysis of cell type specific markers revealed that progenitors grown under control proliferation conditions generate a wave of CNPase-expressing immature oligodendrocytes in response to growth factor withdrawal. After the third day of GFW this CNPase population declines, while the numbers of RIP-, GalC- and MBP-expressing mature oligodendrocytes increase with time (Fig. 5). In contrast to NPCs that were grown under control condition, progenitors pre-treated with MSC-CM persistently generated an increasing number of CNPase-positive cells in response to GFW. In addition, the MSC-CM pre-treatment elevated the numbers of MBP, GalC and RIP positive cells. The decline in the percentage of CNPase expressing cells with a simultaneous increase in MBP, GalC and RIP positive cells in the control condition is somewhat unexpected, since CNPase plays a functional role in oligodendrogenesis and its expression is normally maintained even in mature oligodendrocytes [41-44]. The reason for the decline in CNPase expression in the control condition and 50% MSC-CM treated cells is unclear at present. One attractive hypothesis might be that sustained expression of CNPase might require additional factors present in vivo but not in the control conditions. Nevertheless, MSC might express and secrete such a sustained pro-oligodendroglial activity, since the MSC-CM clearly sustainably elevated the percentage of CNPase expressing cells.

The molecular mechanism of the MSC-CM induced oligodendroglial activity has still to be identified, but it seems to target the regulation of Id proteins. The ratio of the amount of Olig and Id2 proteins is a key determinant for oligodendrocyte versus astrocyte fate decision [19]. In our previous study, we had already demonstrated that MSC-CM induces Olig2 and reduces Id2 expression in neural progenitors that underwent serum-induced differentiation [20]. Under proliferation conditions and serum-free differentiation conditions, MSC-CM did not increase Olig1 and Olig2 expression, but constrained the expression of Id2 and thus raised the Olig2/Id2 ratio. As a consequence, Id2 appears to be a key regulator in the process, and a more detailed analysis of the molecular regulation of Id2 expression might be of interest.

CNS remyelination is the main endogenous regenerative mechanism that protects from demyelination, in particular in the case of MS. Although progenitors are present around MS lesions, migration and differentiation / maturation into myelinating cells is impaired [24,25]. The lack of myelin-promoting activities might contribute to the MS related remyelination impairment. In addition, aging represents a risk factor, since endogenous remyelination is drastically affected in aged subjects [26-28]. First, it has been shown that after demyelination in old rats remyelination occurs only in a slow and delayed fashion [26]. This age-related impairment in remyelination is due to a deficient progenitor recruitment and differentiation [27]. Thus, while remyelination impairment in MS and in particular during aging is partly derived from the failure to differentiate into oligodendrocytes, a lack of sufficient oligodendroglial commitment in proliferating progenitors might contribute to this deficiency. The fact that MSCs not only induce oligodendrocyte differentiation [20] but also prime proliferating NPCs towards an oligodendrocyte fate might have *in vivo* and clinical relevance when aiming to develop remyelinating therapies for MS in young as well as in old patients.

In conclusion, MSCs exert a potent oligodendrogenic effect on adult proliferating NPCs promoting and enhancing cell fate decision, differentiation and maturation. The identification of the MSC derived oligodendrogenic activity as well as the clarification of the underlying molecular mechanism are crucial for the development of new therapies for the treatment of demyelinating diseases such as MS. Moreover, clinical situations that require remyelination of bare axons such as after spinal cord injury might also profit from such a development.

Acknowledgments

The authors would like to thank the following funding agencies for their support: the Bavarian State Ministry of Sciences, Humboldt Stiftung (Georg Forster Program), the Austrian Academy of Sciences (CS; Recipient of a DOC-fFORTE-fellowship of the Austrian Academy of Sciences at the Institute of Molecular Regenerative Medicine), Research and the Arts (ForNeuroCell grant), the Germany Federal Ministry of Education and Research (BMBF grants #01GG0706 and #01GN0979), Deutsche Forschungsgesellschaft (DFG grant #AI31/3-1) and by the state of Salzburg. We disclose any conflict of interest.

References

1. Bunge RP. (1968). Glial cells and the central myelin sheath. Physiol Rev 48:197-251.
2. Chang A, A Nishiyama, J Peterson, J Prineas and BD Trapp. (2000). NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. J Neurosci 20:6404-12.
3. Levine JM, R Reynolds and JW Fawcett. (2001). The oligodendrocyte precursor cell in health and disease. Trends Neurosci 24:39-47.
4. Franklin RJ and MR Kotter. (2008). The biology of CNS remyelination: the key to therapeutic advances. J Neurol 255 Suppl 1:19-25.
5. Bruce CC, C Zhao and RJ Franklin. (2010). Remyelination - An effective means of neuroprotection. Horm Behav 57:56-62.

6. Menn B, JM Garcia-Verdugo, C Yaschine, O Gonzalez-Perez, D Rowitch and A Alvarez-Buylla. (2006). Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 26:7907-18.
7. Gonzalez-Perez O, R Romero-Rodriguez, M Soriano-Navarro, JM Garcia-Verdugo and A Alvarez-Buylla. (2009). Epidermal growth factor induces the progeny of subventricular zone type B cells to migrate and differentiate into oligodendrocytes. Stem Cells 27:2032-43.
8. Aguirre A, JL Dupree, JM Mangin and V Gallo. (2007). A functional role for EGFR signaling in myelination and remyelination. Nat Neurosci 10:990-1002.
9. Jablonska B, A Aguirre, M Raymond, G Szabo, Y Kitabatake, KA Sailor, GL Ming, H Song and V Gallo. (2010). Chordin-induced lineage plasticity of adult SVZ neuroblasts after demyelination. Nat Neurosci 13:541-50.
10. Nait-Oumesmar B, L Decker, F Lachapelle, V Avellana-Adalid, C Bachelin and AB Van Evercooren. (1999). Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination. Eur J Neurosci 11:4357-66.
11. Seri B, DG Herrera, A Gritti, S Ferron, L Collado, A Vescovi, JM Garcia-Verdugo and A Alvarez-Buylla. (2006). Composition and organization of the SCZ: a large germinal layer containing neural stem cells in the adult mammalian brain. Cereb Cortex 16 Suppl 1:i103-11.
12. Rivera FJ, C Steffenhagen, D Kremer, M Kandasamy, B Sandner, S Couillard-Despres, N Weidner, P Kury and L Aigner. (2010). Deciphering the oligodendrogenic program of neural progenitors: cell intrinsic and extrinsic regulators. Stem Cells Dev 19:595-606.
13. Nicolay DJ, JR Doucette and AJ Nazarali. (2007). Transcriptional control of oligodendrogenesis. Glia 55:1287-99.
14. Miller RH. (2002). Regulation of oligodendrocyte development in the vertebrate CNS. Prog Neurobiol 67:451-67.
15. Hu JG, SL Fu, YX Wang, Y Li, XY Jiang, XF Wang, MS Qiu, PH Lu and XM Xu. (2008). Platelet-derived growth factor-AA mediates oligodendrocyte lineage differentiation through activation of extracellular signal-regulated kinase signaling pathway. Neuroscience 151:138-147.
16. Nery S, H Wichterle and G Fishell. (2001). Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain. Development 128:527-40.
17. Orentas DM, JE Hayes, KL Dyer and RH Miller. (1999). Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors. Development 126:2419-2429.
18. Tekki-Kessaris N, R Woodruff, AC Hall, W Gaffield, S Kimura, CD Stiles, DH Rowitch and WD Richardson. (2001). Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. Development 128:2545-54.
19. Samanta J and JA Kessler. (2004). Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development 131:4131-42.
20. Rivera FJ, S Couillard-Despres, X Pedre, S Ploetz, M Caioni, C Lois, U Bogdahn and L Aigner. (2006). Mesenchymal Stem Cells Instruct Oligodendrogenic Fate Decision on Adult Neural Stem Cells. STEM CELLS 24:2209-2219.
21. Rivera FJ, M Kandasamy, S Couillard-Despres, M Caioni, R Sanchez, C Huber, N Weidner, U Bogdahn and L Aigner. (2008). Oligodendrogenesis of adult neural progenitors: differential effects of ciliary neurotrophic factor and mesenchymal stem cell derived factors. J Neurochem 107:832-43.

22. Li QM, YM Fu, ZY Shan, JL Shen, XM Zhang, L Lei and LH Jin. (2009). MSCs guide neurite directional extension and promote oligodendrogenesis in NSCs. Biochem Biophys Res Commun 384:372-7.
23. Rivera F, F Siebzehnrubl, M Kandasamy, S Couillard-Despres, M Caioni, A-M Poehler, B Berninger, B Sandner, B Ulrich, M Goetz, I Bluemcke, N Weidner and L Aigner. (2009). Mesenchymal Stem Cells Promote Oligodendroglial Differentiation in Hippocampal Slice Cultures. Cellular Physiology and Biochemistry In Press.
24. Williams A, G Piaton, MS Aigrot, A Belhadi, M Theaudin, F Petermann, JL Thomas, B Zalc and C Lubetzki. (2007). Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis? Brain 130:2554-65.
25. Solanky M, Y Maeda, X Ming, W Husar, W Li, S Cook and P Dowling. (2001). Proliferating oligodendrocytes are present in both active and chronic inactive multiple sclerosis plaques. J Neurosci Res 65:308-17.
26. Shields SA, JM Gilson, WF Blakemore and RJ Franklin. (1999). Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. Glia 28:77-83.
27. Sim FJ, C Zhao, J Penderis and RJ Franklin. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. J Neurosci 22:2451-9.
28. Hinks GL and RJ Franklin. (2000). Delayed changes in growth factor gene expression during slow remyelination in the CNS of aged rats. Mol Cell Neurosci 16:542-56.
29. Wachs FP, S Couillard-Despres, M Engelhardt, D Wilhelm, S Ploetz, M Vroemen, J Kaesbauer, G Uyanik, J Klucken, C Karl, J Tebbing, C Svendsen, N Weidner, HG Kuhn, J Winkler and L Aigner. (2003). High efficacy of clonal growth and expansion of adult neural stem cells. Lab Invest 83:949-62.
30. Amit M, MK Carpenter, MS Inokuma, CP Chiu, CP Harris, MA Waknitz, J Itskovitz-Eldor and JA Thomson. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227:271-8.
31. Cheng J, A Dutra, A Takesono, L Garrett-Beal and PL Schwartzberg. (2004). Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. Genesis 39:100-4.
32. Smith AG, JK Heath, DD Donaldson, GG Wong, J Moreau, M Stahl and D Rogers. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336:688-90.
33. Livak KJ and TD Schmittgen. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-8.
34. Steffenhagen C, S Kraus, FX Dechant, M Kandasamy, B Lehner, AM Poehler, T Furtner, FA Siebzehnrubl, S Couillard-Despres, O Strauss, L Aigner and FJ Rivera. (2011). Identity, Fate and Potential of Cells Grown as Neurospheres: Species Matters. Stem Cell Rev in press.
35. Trotter J and M Schachner. (1989). Cells positive for the O4 surface antigen isolated by cell sorting are able to differentiate into astrocytes or oligodendrocytes. Brain Res Dev Brain Res 46:115-22.
36. Olstorn H, M Varghese, W Murrell, MC Moe and IA Langmoen. (2011). Predifferentiated brain-derived adult human progenitor cells migrate toward ischemia after transplantation to the adult rat brain. Neurosurgery 68:213-22; discussion 222.
37. Vazey EM and B Connor. (2009). In vitro priming to direct neuronal fate in adult neural progenitor cells. Exp Neurol 216:520-4.

38. Pedraza CE, R Monk, J Lei, Q Hao and WB Macklin. (2008). Production, characterization, and efficient transfection of highly pure oligodendrocyte precursor cultures from mouse embryonic neural progenitors. Glia 56:1339-1352.
39. Gibney SM and KW McDermott. (2007). Differentiation of oligodendrocytes in neurospheres derived from embryonic rat brain using growth and differentiation factors. J Neurosci Res 85:1912-20.
40. Palmer TD, J Ray and FH Gage. (1995). FGF-2-Responsive Neuronal Progenitors Reside in Proliferative and Quiescent Regions of the Adult Rodent Brain. Molecular and Cellular Neuroscience 6:474-486.
41. de Castro F and A Bribian. (2005). The molecular orchestra of the migration of oligodendrocyte precursors during development. Brain Res Brain Res Rev 49:227-41.
42. McMorris FA, SU Kim and TJ Sprinkle. (1984). Intracellular localization of 2',3'-cyclic nucleotide 3'-phosphohydrolase in rat oligodendrocytes and C6 glioma cells, and effect of cell maturation and enzyme induction on localization. Brain Res 292:123-31.
43. Domanska-Janik K, B Gajkowska, B de Nechaud and JM Bourre. (1988). Myelin composition and activities of CNPase and Na+,K+-ATPase in hypomyelinated "pt" mutant rabbit. J Neurochem 50:122-30.
44. Scherer SS, PE Braun, J Grinspan, E Collarini, DY Wang and J Kamholz. (1994). Differential regulation of the 2',3'-cyclic nucleotide 3'-phosphodiesterase gene during oligodendrocyte development. Neuron 12:1363-75.

Figure Legends

FIG. 1. The oligodendrogenic process of neural stem / progenitor cells and the experimental design. A) In the oligodendroglial lineage, undifferentiated neural stem cells (NSCs) undergo sequential steps of cell lineage commitment, differentiation, and maturation involving different cell types and culminating in myelinating oligodendrocytes (OLs). Due to the specific marker expression profile each cell type can be identified during this process. Proliferating (circular small arrow) Nestin-expressing NSCs, follow a specification step to give rise to glial progenitor cells (GPCs), characterized by the expression of A2B5 epitope, platelet derived growth factor receptor-alpha (PDGFRα) and the chondroitin sulfate proteoglycan NG2. In turn, proliferating GPCs can give rise to O4-expressing cycling oligodendrocyte progenitor cells (OPCs) that undergo glial fate decision towards astrocytes or OLs. Several glial fate determinants regulate this step, in particular the relative expression levels of Oligs and Ids play an important role at the glial branch point. A high Oligs / Id2 ratio implies an oligodendrocyte fate decision and vice versa. Therefore, cell lineage commitment and restriction might occur in proliferating immature stem / progenitor cells. Next OPCs follow a differentiation process towards immature 2',3'-cyclic nucleotide 3' phosphodiesterase- (CNPase) and RIP-expressing OLs. Finally, a maturation process takes place and will give rise to myelinating OLs, characterized by the expression of Galactocerebroside (GalC) and myelin basic protein (MBP). **B)** The experimental of the present work uses a three week (3W) treatment of proliferating (circular small arrow) NPCs with MSC-CM. During this period, cell morphology and proliferation are followed. Proliferating NPCs give rise to neurospheres that are dissociated for subsequent experiments and analyses. Cell identity is analyzed directly after the three week stimulation by profiling cell type specific marker expression; the intrinsic differentiation fate of the cells is analyzed after a one week (1W) period of growth factor withdrawal (GFW);

the oligodendroglial cell lineage restriction is tested by exposing the primed cells for 1W to serum, an astroglia inducing activity; finally, the expression of Olig2 and Id2 is determined in proliferating NPCs directly after the 3W of exposure to MSC-CM.

FIG. 2. Morphology and growth curve of proliferating NPCs. Phase contrast images of NPCs in normal proliferation medium (control) **(A, E, I)**, treated with 50% MSC-CM **(B, F, J)**, 100% MSC-CM **(C, G, K)** or MSC-CM replenished with growth factors (MSC-CM + GF) **(D, H, L).** Proliferating NPCs were incubated for 7 days **(A-D)**, 14 days **(E-H)** and 21 days **(I-L)**. Note that under control conditions proliferating NPCs grew as floating aggregates, but when the cells were treated with MSC-CM some NPCs attached to the surface of the flask and grew adherent. Scale bar = 100 µm. The cell number was determined by Trypan blue exclusion after 7, 14 and 21 days. Growth curves for the different conditions were determined (**M**). The metabolic activity of the cells **(N)** as well as the number of neurospheres **(O)** and the mean neurospheres diameter **(P)** were analyzed on day 2, 4 and 7 during the second week of incubation with the respective media. Note that there is no significant effect of MSC-CM on the NPCs proliferation rate. All experiments were performed at least in triplicate in three independent experiments. Data are shown as mean +/- SD. For statistical analysis two-way ANOVA-Bonferroni post-hoc was performed.

FIG. 3. Cell identity: marker expression profile of pre-treated proliferating NPCs. NPCs were dissociated after the 3W exposure to MSC-CM or control conditions, and cells were seeded overnight under serum-free conditions. The marker expression profile was analyzed by immunocytochemistry and quantitative RT-PCR. Illustrative fluorescence images of the immunostainings are shown for: Nestin (red) and DAPI (blue) **(A)**; A2B5 (red) and DAPI (blue) **(C)**; PDGFRα (green) and DAPI (blue) **(E)**; NG2 (green) and DAPI **(G)**; and O4 (red) and DAPI (blue) **(I)**; RIP (red) and DAPI (blue) **(K)**; CNPase (red) and DAPI (blue) **(M)**; GalC

(green) and DAPI (blue) **(O)**; MBP (red) and DAPI (blue) **(Q)**; GFAP (green) and DAPI (blue) **(S)**; DCX (green) and DAPI (blue) (**K**). Scale bar = 10µm. Quantitative analysis show the percentage of positive cells for each marker (**B, D, F, H**, **J**, **L, N, P, R, T and V**). Note that the number of RIP-positive cells was significantly increased after MSC-CM treatments compared to control condition, while there was no significant change for the other tested markers after MSC-CM stimulation. **(W)** Quantitative RT-PCR for glial fate determinants and stem / progenitor cell markers. Delta delta Ct method was used for analysis considering glyceraldehyde 3-phosphate dehydrogenase as normaliser gene and control condition as calibrator. Relative expression levels of Nestin, NG2, PDGFRα, Olig1, Olig2, CNPase and MBP after treatment with 100% MSC-CM compared to control condition (dashed line) are shown. After 3 weeks of pre-treatment with MSC-CM there was a significant increase in the expression of CNPase and MBP, while there was a slight decrease in the expression levels of NG2 and PDGFRα. All experiments were performed at least in triplicate in three independent experiments. Data are shown as mean +/- SD. For statistical analysis one-way ANOVA-tukey post-hoc was performed. Asterisks above individual columns indicate significant difference compared with control. Asterisks above a line spanning two columns indicate significant difference between MSC-CM treatments. * = $p<0.05$; *** = $p<0.001$.

FIG. 4. Cell intrinsic differentiation fate: GFW response of pre-treated proliferating NPCs. NPCs that were exposed under proliferation conditions to MSC-CM or control conditions were dissociated, seeded and incubated for one week under serum-free conditions in the absence of growth factors. Cells were fixed and the expression of cell-specific-lineage markers and fate determinants was analyzed by immunofluorescence and quantitative RT-PCR. Illustrative fluorescence images are shown for: O4 (red) and DAPI (blue) **(A)**; RIP (red) and DAPI (blue) **(C)**; CNPase (red) and DAPI (blue) **(E)**; GalC (green) and DAPI (blue) **(G)**; MBP (red) and DAPI (blue) **(I)**; GFAP (green) and DAPI **(K)**; DCX (green) and DAPI (blue)

(M); and MAP2ab (red) and DAPI (blue) **(O)**. Scale bar = 10µm. Quantitative analysis show the percentage of positive cells for each marker (**B, D, F, H, J**, **L, N and P**). Note the significant increase in the percentage of cells that express the oligodendroglial markers RIP, CNPase, GalC and MBP when proliferating NPCs were pre-treated with MSC-CM. **(Q)** Quantitative RT-PCR for glial fate determinants and oligodendrocyte markers. Delta delta Ct method was used for analysis considering glyceraldehyde 3-phosphate dehydrogenase as normaliser gene and control condition as calibrator. Relative expression levels Olig1, Olig2, Id2, CNPase and MBP after treatment with 100% MSC-CM compared to control condition (dashed line) are shown. All experiments were performed at least in triplicate in three independent experiments. Data are shown as mean +/- SD. For statistical analysis one-way ANOVA-tukey post-hoc was performed. Asterisks above individual columns indicate significant difference compared with control. Asterisks above a line spanning two columns indicate significant difference between MSC-CM treatments. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

FIG. 5. Cell intrinsic differentiation fate: temporal expression of neural markers of pre-treated proliferating NPCs in response to GFW. NPCs were grown for three weeks in the presence of MSC-CM, dissociated and seeded overnight under serum-free conditions. Then, the cells were either fixed to analyze the marker expression profile at day 0 or the cells were incubated in the absence of growth factors for 3 and 7 days and fixed to analyze temporal changes in the marker expression profile. Immunofluorescence was performed for the presence of differentiating and mature oligodendrocyte-, as well as for astrocyte- and neuron- specific markers. Quantitative analysis show over-time the percentage of cells that express: MBP **(A)**, GalC **(B)**, RIP **(C)**, CNPase **(D)**, GFAP **(E)** and DCX **(F)**. NPCs pre-treated with MSC-CM display more MBP-, GalC-, RIP- and CNPase-expressing cells along time than untreated NPCs after growth factor withdrawal. All experiments were performed at least in triplicate in three independent experiments. Data are shown as mean +/- SD. For statistical analysis two-

way ANOVA-Bonferroni post-hoc was performed. Asterisks indicate significant difference compared with control. ** = $p<0.01$; *** = $p<0.001$.

FIG. 6. Cell fate restriction: neural differentiation of pre-treated proliferating NPCs in response to astrogenic conditions. NPCs treated for 3W under proliferation conditions with or without MSC-CM were dissociated, seeded and incubated for one week in the presence of FBS as an astrogenic stimulus. Cells were fixed and cell-specific-lineage marker expression was analyzed by immunofluorescence. Quantitative analysis show the percentage of cells that express: MBP **(A)**, CNPase **(B)**, GFAP **(C)** and DCX **(D)**. Note that in despite of astrogenic conditions, NPCs pre-treated with MSC-CM display less GFAP-expressing astrocytes and more MBP- as well as CNPase- expressing oligodendrocytes than non pre-treated NPCs. All experiments were performed at least in triplicate in three independent experiments. Data are shown as mean +/- SD. For statistical analysis one-way ANOVA-tukey post-hoc was performed. Asterisks above individual columns indicate significant difference compared with control. Asterisks above a line spanning two columns indicate significant difference between MSC-CM treatments. * = $p<0.05$; *** = $p<0.001$.

FIG. 7. Glial fate determinants expression of pre-treated proliferating NPCs. Proliferating NPCs were grown for 3 weeks under normal conditions (control), 50% or 100% MSC-CM. Quantitative RT-PCR was performed to analyze the expression of the pro-oligodendrogenic transcription factor Olig2 and the anti-oligodendrogenic determinant, Id2. Delta delta Ct method was used considering glyceraldehyde 3-phosphate dehydrogenase as normaliser gene and control conditions as a calibrator. Expression levels of Olig2 (A) and Id2 (B) under the different conditions are shown. To determine Olig2/Id2 ratio, Olig2 expression levels were quantified using Id2 as a calibrator gene in the respective condition (C). Note that while MSC-CM does not affect Olig2 expression it strongly decreases Id2 levels. MSC-CM-

treated proliferating NPCs displays 6 to 7 fold increase on the Olig2/Id2 ratio compared to control conditions. All experiments were performed at least in triplicate in three independent experiments. Data are shown as mean +/- SD. For statistical analysis one-way ANOVA-tukey post-hoc was performed. Asterisks above individual columns indicate significant difference compared with control. Asterisks above a line spanning two columns indicate significant difference between MSC-CM treatments. ** = $p<0.01$; *** = $p<0.001$.

Curriculum vitae

Name:	DECHANT, Franz – Xaver
Date of birth:	July 4, 1986
Place of birth:	Regensburg
Citizenship:	German
Address:	Hoher – Kreuz – Weg 32 D- 93055 Regensburg Germany
Marital status:	single
Parents:	Father: Franz – Josef Dechant Mother: Bernadette Dechant,

EDUCATION

Primary Education:	1992-1996 Grundschule (primary school) Hohes Kreuz, Regensburg
Secondary Education:	1996 – 2005 Musikgymnasium der Regensburger Domspatzen (graduation: Abitur)
Academic Studies:	Since 2005 study of human medicine at the University of Regensburg Since 2008, Conferral of a doctorate at the department of neurology of the University of Regensburg (doctoral advisor Prof.Dr.rer.nat. Ludwig Aigner)
Work Experience:	July 4- September 2, 2005 and July 31-August 30, 2006 practical training at the St. Josef Hospital, Regensburg February 18- march 16, 2008 clinical elective at the surgical doctor's practise of Dr. med Schindler and Dr. med. Gruber, Regensburg August 4-31, 2008 clinical elective at the department of anaesthesia (Hospital of the order of the Merciful Brothers, Vienna) August 3-17, 2009 clinical elective at the department of internal medicine (Hetzelstift Hospital, Neustadt/ Weinstraße, Germany) August 24-September 22, 2009 clinical elective at the urology department (Ospedale San Giovanni in Laterano, Rome)

September 28-october 12, 2009 clinical elective at the nephrology department (St. Josef hospital, Regensburg)
August 30 – October 24, 2011 practical year in the department of surgery (university hospital, Regensburg)
October 25 – December 17, 2011 practical year at the department of transplant surgery (Guy's Hospital, university of London)
December 20 – April 8, 2011 Practical year at the department of urology (St. Josef hospital, Regensburg)
April 11 – July 29, 2011 Practical year at the department for internal medicine (Hospital of the order of the Merciful Brothers, Regensburg)

Language Skills: English (seven years), Latin (seven years), Ancient-Greek (three years), Italian (three years), Chinese (one year)

Acknowledgements

At the end of this thesis I want to thank all people, who helped and supported me during my work.

First I want to thank Prof. Ludwig Aigner for giving me the opportunity to make my thesis in his laboratory.

Especially I want to thank my supervisor Dr. Francisco Rivera for providing me this interesting topic for my thesis, for always encouraging me and for helping me with scientific questions.

Also I want to thank all my correctors and all people who supported me in writing this thesis. This people are in detail: Dr. Martin Drage M.D, university of London, Jayne Bell, Dr. Krish Menon, Rebecca Mayr, Anna Grigorakakis and Carola Schum.

Further I want to thank all my helpful colleges for the nice and friendly atmosphere and the helpful discussions during my work. In detail I thank Carolin Steffenhagen, Bernadette Lehner, Beatrice Sandner, Kathrin Stadler, Massimliano Caioni and Dirkje Felder.

Finally, I want to thank my girlfriend Rebecca Mayr, my parents Bernadette and Franz – Josef Dechant as well as my siblings Maria and Martin Dechant for always supporting me and giving me the opportunity to fulfil my studies in medicine.

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt

Regensburg, den 27. Oktober 2011 .....

Franz – Xaver Dechant