Heinrich-Heine-Universität Düsseldorf

Identification of mesenchymal stem cell-derived factors with pro-oligodendrogenic activities

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To my family

„Wir geben niemals auf, öffnen neue Türen
und entwickeln neue Ideen, weil wir neugierig sind…
Die Neugier ist es, die uns neue Wege beschreiten lässt“

W. D.

1. Summary / Zusammenfassung

1.1 Summary

Multiple sclerosis (MS) is one of the most common demyelinating diseases in which autoimmune activities cause a depletion of oligodendrocytes and myelin sheaths resulting in impaired electric signal transduction. During disease progression, myelin repair activities can be observed as a consequence of activation, recruitment and differentiation of resident stem- and precursor cells such as oligodendroglial progenitor cells (OPCs) and subventricular zone (SVZ) derived resident multipotent adult the neural stem cells (aNSCs). However, the overall degree of remyelination and functional regeneration is limited due to maturation failure and presence of inhibitory factors preventing the interaction of myelin-forming cells with axons. Further, the immune response appears to exert an additional negative impact on tissue regeneration and repair. Therefore, specific stimulation of aNSCs to differentiate into the oligodendroglial cell lineage could be a promising overall strategy to promote regeneration with special emphasis on myelin repair. In this regard, mesenchymal stem cell (MSC)-secreted factors have previously been shown to enhance oligodendrogenesis of cultured primary aNSCs. However, the functional degree of MSC depending lineage instruction *in vivo* remained to be established. Furthermore, MSC-secreted factors fostering an oligodendroglial differentiation have not been identified so far. The focus of the present study was therefore the identification of corresponding MSC-secreted compounds and the analysis of MSC-conditioned medium (MSC-CM) stimulated aNSCs *in vivo*.

It was revealed that MSC-CM pre-stimulated aNSCs transplanted into different regions of the rodent CNS predominantly differentiate into the oligodendroglial lineage *in vivo*. This effect was observed independent of grey or white matter localization and the pre-stimulation period. The overall degree of surviving cells after transplantation was reduced over time and differed between species and CNS regions. To identify MSC-secreted pro-oligodendroglial proteins, a quantitative mass spectrometry analysis of the MSC secretome was performed resulting in 152 enriched proteins whereof 16 proteins were known to affect oligodendroglial differentiation. Two candidate proteins, chordin and the tissue inhibitor of metalloproteinase 1 (TIMP-1), were further investigated in culture by neutralization of the respective protein in MSC-CM before application to aNSCs. While blocking of

chordin showed no effect on the oligodendroglial differentiation of cultured aNSCs, TIMP-1 neutralization led to a significantly reduced oligodendroglial cell differentiation. Furthermore, TIMP-1 neutralization also diminished the pro-oligodendroglial effect of MSC-CM on aNSCs transplanted into the spinal cord.

Moreover, for biomedical translation, the secretome derived from human iPSCs-derived MSCs could be an attractive option to replace bone marrow stem cells. Indeed, stimulation of cultured aNSCs with conditioned medium from human iPSC-derived MSCs revealed a strong increase of oligodendroglial marker expression at the expense of the astroglial marker expression. Summarized, the present data demonstrated a strong impact of rodent and human MSC-secreted factors on the oligodendroglial lineage differentiation of aNSCs in culture as well as *in vivo* and revealed candidate proteins likely to be responsible for these effects.

1.2 Zusammenfassung

Multiple Sklerose (MS) ist die am häufigsten auftretende demyelinisierende Krankheit des Zentralnervensystems (ZNS), bei der eine Autoimmunreaktion zum Verlust von Oligodendrozyten und Myelinscheiden führt, welches eine eingeschränkte elektrische Signalweiterleitung zur Folge hat. Während des Krankheitsverlaufs können Myelin-Reparatur-Aktivitäten beobachtet werden als Konsequenz einer Aktivierung, Rekrutierung und Differenzierung von Stamm- und Vorläuferzellen wie oligodendroglialer Vorläuferzellen (OPCs) sowie residenter multipotenter adulter neuraler Stammzellen (aNSCs) der subventrikuläre Zone (SVZ). Der Gesamtgrad der Remyelinisierung und funktionellen Regeneration ist jedoch begrenzt, was auf intrinsische Reifungsdefizite der Zellen oder inhibitorische Faktoren zurückzuführen ist, die beispielsweise die Interaktion zwischen myelinisierenden Zellen und Axon verhindern oder die Reifung von OPCs negativ beeinflussen. Des Weiteren scheint die Immunantwort einen zusätzlichen negativen Einfluss auf die Geweberegeneration und -reparatur auszuüben. Daher könnte eine gezielte Stimulation von aNSCs zur Steigerung der oligodendroglialen Differenzierung ein insgesamt erfolgsversprechender Behandlungsansatz zur Förderung der Remyelinisierung sein. In diesem Zusammenhang wurde bereits gezeigt, dass von mesenchymalen Stammzellen (MSCs) sekretierte Faktoren die Oligodendrogenese von kultivierten primären aNSCs verbessern. Die Funktionalität der MSC-abhängigen oligodendroglialen Differenzierung wurde bislang jedoch *in vivo* nicht untersucht. Darüber hinaus sind die MSC-sekretierten Faktoren, die eine oligodendrogliale Differenzierung fördern, weitestgehend unbekannt. Der Fokus der vorliegenden Studie lag daher auf der Identifizierung entsprechender MSC-sezernierter Faktoren und der Analyse von MSC-konditionierten Medium (MSC-CM) stimulierter aNSCs *in vivo*. Es zeigte sich, dass in verschiedene ZNS-Regionen transplantierte aNSCs, die zuvor mit MSC-sezernierten Faktoren stimuliert wurden, bevorzugt in oligodendrogliale Zelltypen differenzieren. Dieser Effekt konnte unabhängig von der Lokalisation in grauer oder weiße Substanz sowie Stimulationszeitraum mit MSC-sezernierten Faktoren gezeigt werden. Allerdings war eine Zeitpunkt- und Spezies-abhängige Reduktion der Überlebensrate der transplantierten Zellen zu verzeichnen. Zur Identifizierung MSC-sekretierter pro-oligodendroglialer Proteine, wurde eine quantitative massenspektrometrische Analyse des MSC-Sekretoms durchgeführt, bei der 152 Proteine identifiziert wurden, von denen 16 Proteine bekanntlich einen

Einfluss auf die oligodendrogliale Differenzierung haben. Der Effekt der Kandidatenproteine Chordin und *tissue inhibitor of metalloproteinase 1* (TIMP-1) auf die Oligodendrogenese wurde durch Neutralisierung des jeweiligen Proteins in MSC-CM näher untersucht. Während die Blockierung von Chordin keine Auswirkungen auf die oligodendrogliale Differenzierung kultivierter aNSCs hatte, führte die TIMP-1-Neutralisierung zu einer signifikanten Reduktion des pro-oligodendroglialen Effekts. Zudem war der pro-oligodendrogliale Effekt von MSC-CM auf transplantierte NSCs durch Neutralisation von TIMP-1 ebenso deutlich reduziert. Darüber hinaus könnten für eine spätere biomedizinische Anwendung sezernierte Faktoren humaner von iPSC abstammender MSCs als Ersatz für MSCs aus Knochenmark dienen. Die Stimulation kultivierter aNSCs mit sezernierten Faktoren von humanen iPSC-abgeleiteten MSCs führte zu einer vergleichbaren Erhöhung der oligodendroglialen, bei gleichzeitiger Reduktion der astroglialen Markerexpression. Zusammengefasst konnte durch die vorliegende Arbeit ein starker Einfluss von Nager- und humanen MSC-sezernierten Faktoren auf die oligodendrogliale Differenzierung von aNSCs sowohl in Kultur als auch *in vivo* nachgewiesen werden. Zudem wurden mögliche Kandidatenproteine identifiziert, die für diese Effekte verantwortlich sind.

2. Introduction

2.1 Stem cells

Stem cells are characterized by two major functions, the ability to self-renew and their potential to differentiate, and can be found in the entire organism (Figure 2.1). Self-renewal is mediated by the tissue environment which allows stem cells to undergo a symmetric cell division to give rise to two identical daughter cells (Morrison and Kimble, 2006; Alberts et al., 2008). For differentiation, stem cells divide asymmetrically and generate two different daughter cells (Knoblich, 2008; Gilbert, 2014) giving rise to one cell maintaining mother stem cell properties whereas the other cell differentiates into a more mature cell (Alberts et al., 2008; Knoblich, 2008; Gilbert, 2014).

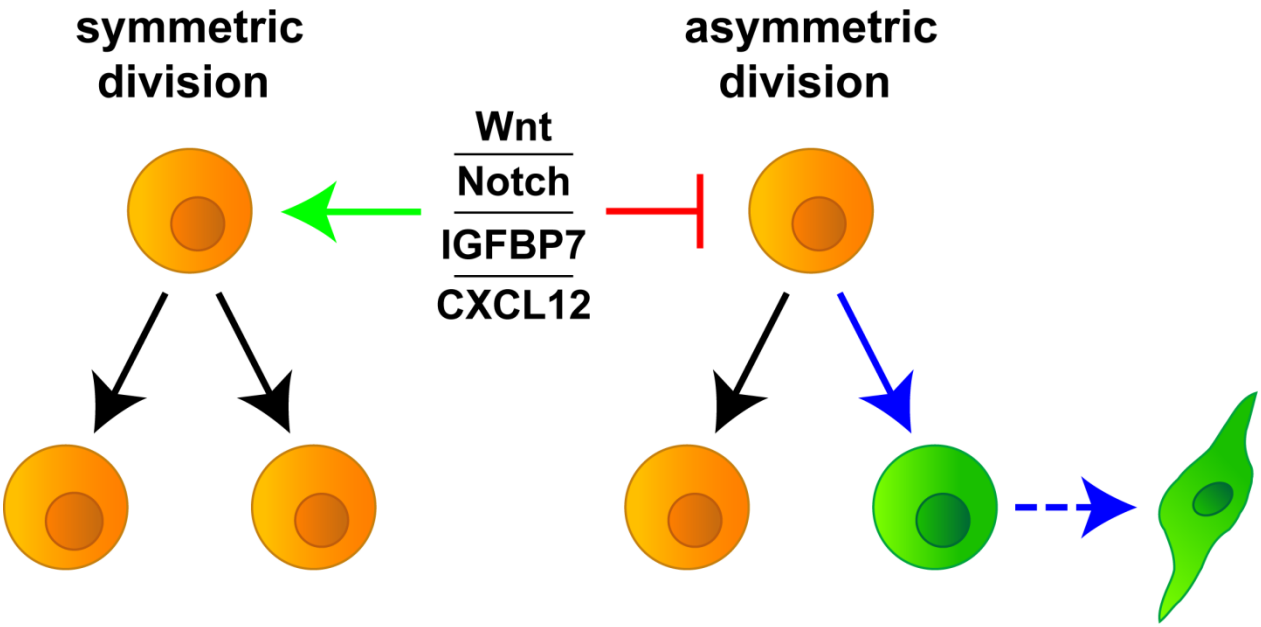

Figure 2.1: Symmetric and asymmetric stem cell division

Stem cell renewal and differentiation are regulated by niche depending signals. Each stem cell type has its individual niche, which in turn regulates stem cell properties in an autocrine manner by secretion of different chemokines e.g., C-X-C motif chemokine ligand 12 (CXCL12) activates the C-X-C motif chemokine receptor 4 (CXCR4) which promotes differentiation of embryonic stem cells (Zhang et al., 2016), whereas CXCL12/CXCR4 promotes proliferation of endothelial stem cells in injured tissue (Bianchi and Mezzapelle, 2020) or proteins like insulin-like growth factor-binding proteins 7 (Igfbp7) controls through Wnt-signaling the asymmetric division of mesenchymal stem cells (Zhang et al., 2018a). Furthermore, activation of membrane bound receptors via Wnt or Notch signaling controls the maintenance and differentiation of the stem cell pool of, e.g., hematopoietic stem cells (Calvi et al., 2003; Reya and Clevers, 2005), lung stem cells (Xu et al., 2018) or neural stem cells (Aguirre et al., 2010; Chavali et al., 2018).

The stem cell microenvironment, also known as ‘stem cell niche’, regulates and maintains stem cell division as well as cell polarity and fate by the secretion of various signaling molecules (Alberts et al., 2008), e.g., wingless-related integration site (Wnt), Notch or sonic hedgehog (Shh), membrane-bound factors such as stem cell factor (SCF), and chemokines like CXCL12 (Figure 2.2) (Li and Xie, 2005; Palma et al., 2005; Reya and Clevers, 2005; Katayama et al., 2006; Lane et al., 2014; Vishwakarma and Karp, 2017; Xu et al., 2018; Bianchi and Mezzapelle, 2020). It should be noted that each stem cell niche is individually adapted to the respective stem cell type, however, different signaling molecules have unique functions in different stem cell niches such as Wnt and Notch. The inhibition of Wnt, e.g., effectively stimulates asymmetric cell division of lung stem cells (Xu et al., 2018). Furthermore, Wnt also control the stem cell maintenance of hematopoietic, stoma and follicle cells (Reya and Clevers, 2005) and regulates neural stem cell quiescence (Chavali et al., 2018). Notch is necessary for symmetric cell division while the inhibition of Notch triggers the switch towards an asymmetric cell division in drosophila (Egger et al., 2010). In addition, Notch is involved in the regulation of asymmetric cell division in mice intestine cells (Srinivasan et al., 2016) and influences the number of hematopoietic stem cells (Calvi et al., 2003). Beside Wnt and Notch, Shh plays an important role in various stem cell niches. In the mouse forebrain subventricular zone stem cell niche, Shh is required for cell proliferation and the generation of new olfactory interneurons (Palma et al., 2005). Furthermore, Shh can control the number and the location of hair follicles in the adult epidermis (Silva-Vargas et al., 2005). Beside of these very common signaling molecules also other molecules, chemokines and growth factors are involved in the maintenance and regulation of stem cells in diverse niches. SCF as membrane-bound factor is a key regulator in the initial accumulation of hematopoietic stem cells and has been shown to play a role in cell mobilization and migration (Driessen et al., 2003). The chemokine CXCL12 regulates proliferation, stem cell maintenance, differentiation as well as cell migration. The secretion of CXCL12, e.g., promotes proliferation of endothelial stem cells in injured tissue (Bianchi and Mezzapelle, 2020) whereas in naïve tissue CXCL12 promotes the differentiation of embryonic stem cells through the activation of CXCR4 (Zhang et al., 2016). In addition, CXCL12 signaling in the bone marrow niche can mediate the migration as well as the maintenance of hematopoietic stem cells (Greenbaum et al., 2013). Furthermore, IGFBP7 controls, asymmetric cell

division of mesenchymal stem cells towards an osteogenic differentiation through Wnt-signaling (Zhang et al., 2018a).

A key component of the stem cell niche is the extracellular matrix (ECM) composed of different proteins depending on the niche type (Watt and Huck, 2013; Lane et al., 2014). ECM proteins are important for orientation and structural maintenance of the stem cell niche (Gattazzo et al., 2014; Lane et al., 2014; Vishwakarma and Karp, 2017). The interaction between the ECM and stem cells is mediated by a number of cell receptors including integrins (Gattazzo et al., 2014) which in turn interact with other signaling molecules. For neural stem cells (NSCs) and mammary stem cells, β1-integrin was shown to regulate self-renewal and differentiation by controlling the activity of Notch and epidermal growth factor (EGF) receptor (Campos et al., 2006; Brisken and Duss, 2007).

The impact of the ECM on stem cells further depends the glycoprotein tenascin C (TNC) which is known to play a role in certain stem cell niches affecting proliferation and differentiation of neural, epithelial and osteogenic stem cells (Garcion et al., 2001; Tucker et al., 2013; Chiquet-Ehrismann et al., 2014). In hematopoiesis, TNC controls lineage commitment and cell migration of T cells (Ellis et al., 2013). Another stem cell niche regulator Ca^{2+} known to be involved in osteogenic differentiation of mesenchymal stem cells (Nakamura et al., 2010).

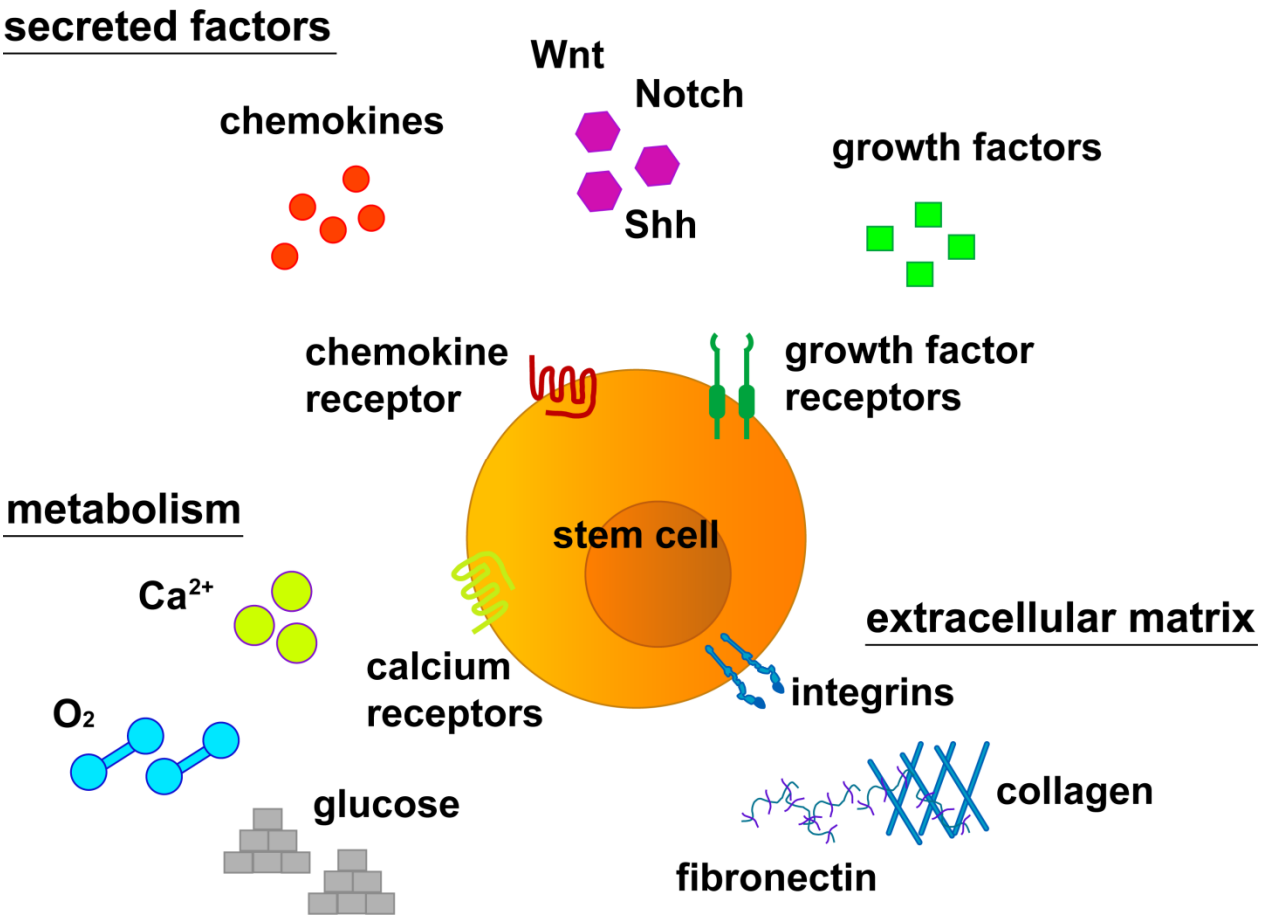

Figure 2.2: Stem cell niche factors regulating the maintenance and function of stem cells

The stem cell niche regulates cell division and cell fate by paracrine mechanisms, cell-cell contact as well as metabolic products. Each stem cell niche is unique and individually adapted to the corresponding stem cell type and the environment. Nevertheless, many properties are common, e.g., secreted factors such as Wnt or Notch, growth factors or chemokines binding to surface receptors regulating cell fate and self-renewal. Furthermore, ECM compounds are important for the interaction of stem cells with the environment and structural maintenance of the niche. Furthermore, stem cell maintenance and differentiation are regulated by metabolic compounds (Lane et al., 2014).

In general, the differentiation potential differs between stem cells depending on the origin as well as on the developmental stage of the tissue or organism. Omnipotent or totipotent stem cells derived from a zygote are able to differentiate into cells of a whole organism whereas embryonic stem cells (ESCs) are pluripotent stem cells which differentiate into almost every cell type of the three germ layers except the placenta (Evans and Kaufman, 1981; Thomson et al., 1998; Alberts et al., 2008). Since ESCs are isolated from blastocysts of the fertilized ovum (Thomson et al., 1998) rising ethical concerns, induced pluripotent stem cells (iPSCs) have opened a new opportunity in the field of disease modeling and studying diseases (Takahashi and Yamanaka, 2006; Halevy and Urbach, 2014) which are morphologically and

genetically almost identical to ESCs (Takahashi et al., 2007a). In contrast, adult or multipotent stem cells display a smaller differentiation potential giving rise to cells one of a specific germ layer (Alberts et al., 2008). Mesenchymal stem cells (MSCs) (Bruder et al., 1997) for example, are able to differentiate into osteoblasts, chondrocytes, adipocytes, fibroblasts and myoblasts (Friedenstein et al., 1966; Friedenstein et al., 1970; Friedenstein et al., 1974; Friedenstein, 1976; Friedenstein et al., 1976; Friedenstein et al., 1978; Bruder et al., 1997; Kadiyala et al., 1997; Ferrari et al., 1998; Young et al., 1998; Dennis et al., 1999) whereas hematopoietic stem cells (HSCs) (Alberts et al., 2008; Birbrair and Frenette, 2016) give rise to all blood cell types (Alberts et al., 2008; Birbrair and Frenette, 2016). Neural stem cells (NSCs) (Clarke et al., 2000; Beattie and Hippenmeyer, 2017) generate neurons and glial cells of the central nervous system (CNS) (Kriegstein and Alvarez-Buylla, 2009). Stem cells with the potential to differentiate only into a few cell types of a specific tissue type are oligopotent cells, e.g., lymphoid or myeloid cells (Alberts et al., 2008). In addition, cells only giving rise to one specific cell type like hepatoblasts (Fuchs et al., 2004; Alberts et al., 2008) are known as unipotent stem cells.

2.1.1 Neural stem cells

During telencephalon development, neuroepithelial cells generate radial glial cells (RGs) (Doetsch, 2003; Kriegstein and Alvarez-Buylla, 2009). The long processes of RGs extend from the apical to the basal side of the cortex and serve as a guide for newly generated neurons in the ventricular zone (VZ) to distant locations within the telencephalon (Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). The neuroepithelial origin of RGs is reflected by their common marker expression such as the neuroectodermal stem cell marker (nestin) and intermediate filament-associated protein (RC2) (Lendahl et al., 1990; Hartfuss et al., 2001; Mori et al., 2005; Gilyarov, 2008; Park et al., 2009; Park et al., 2010). However, RGs also differ from neuroepithelial cells by expressing the astroglial markers glutamate aspartate transporter (GLAST) and brain lipid binding protein (Blbp) (Feng et al., 1994; Malatesta et al., 2000; Hartfuss et al., 2001; Mori et al., 2005). The potential to express neuroepithelial and astroglial markers indicate that RGs are a heterogeneous cell population (Kriegstein and Götz, 2003), thus consisting of various subpopulations (Hartfuss et al., 2001). RGs are known to divide asymmetrically to give rise to neurons or intermediate progenitor cells (IPCs) (Figure 2.3) which generate and

colonize the subventricular zone (SVZ) and in turn can also generate neurons (Haubensak et al., 2004; Noctor et al., 2004). IPCs consist of different subtypes including neurogenic intermediate progenitor cell (nIPCs) which generate neurons and oligodendrocytic intermediate progenitor cell (oIPCs) differentiating into mature oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009). Both IPCs types emerge from early RGs while astrocytes emerge from late RGs. At the end of embryonic development, most of the RGs lose their contact to the apical cortex side and differentiate into astrocytes which migrate to the cortical plate while oIPC formation continues.

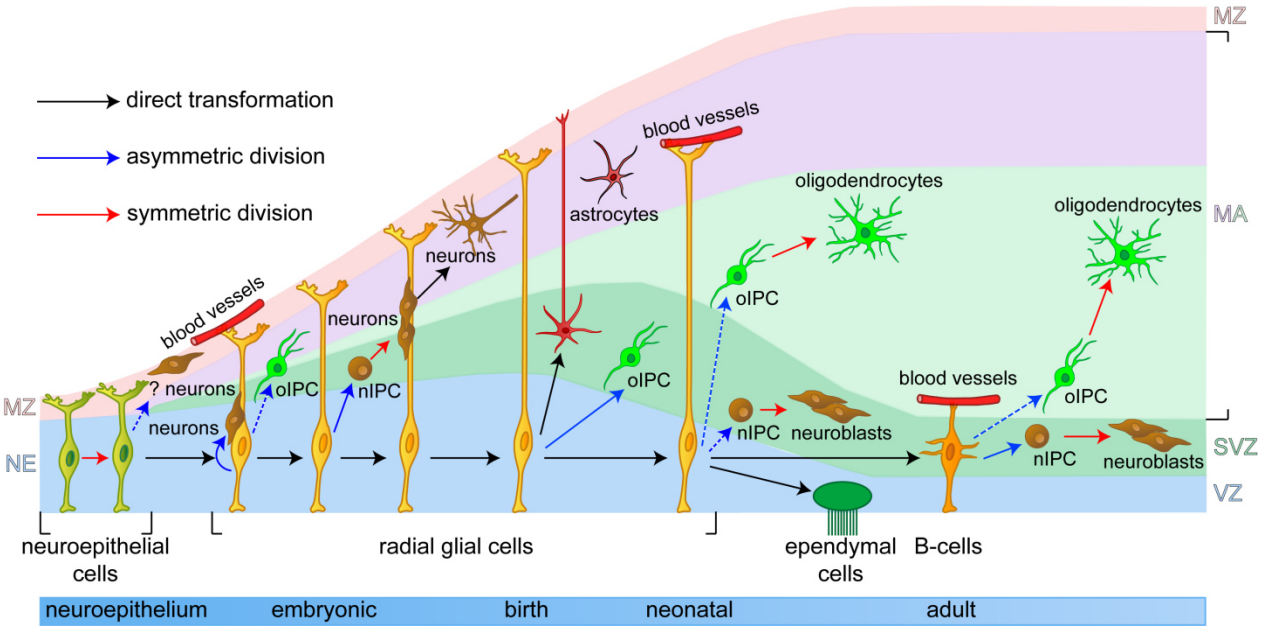

Figure 2.3: NSCs, radial glia cells and IPCs in the developing and adult telencephalon

Neuroepithelial cells generate radial glial cells. RGs divide asymmetrically and can directly and indirectly generate neurons via IPCs. In addition, there are different types of IPCs which give rise to astrocytes, neurons or oligodendrocytes, respectively. At the end of development, most of the RGs lose their contact to the apical cortex side and differentiate into astrocytes. In the adult cortex, RGs remaining in contact with the apical side are neural stem/progenitor cells which are defined as adult SVZ astrocytes called B-cells. B-cells generate C-cells (transit-amplifying progenitors; TAPs), which in turn generate A-cells (neuroblasts). MA: mantle, MZ: marginal zone, NE: neuroepithelium, nIPCs: neurogenic intermediate progenitor cell, oIPCs: oligodendrocytic intermediate progenitor cell, RGs: radial glia cells, SVZ: subventricular zone, VZ: ventricular zone. Adapted from Kriegstein and Alvarez-Buylla, 2009.

The cortical development continues until postnatal stages, in with the SVZ contains resident RG descending neural stem cells (NSCs), called B-cells, until adulthood (Figure 2.3; 2.4). B-cells are derived from the RGs and are also known as adult SVZ astrocytes (Merkle et al., 2004; Kriegstein and Alvarez-Buylla, 2009) expressing astroglial markers such as glial fibrillary acidic protein (GFAP) and GLAST (Doetsch

et al., 1997; Colak et al., 2008; Platel et al., 2009) as well as the stem cell markers nestin and sex determining region Y-box transcription factor (Sox) 2 (Weiss et al., 1996). B-cells are known to generate active proliferating type C-cells, also called transit-amplifying progenitors (TAPs), which act as intermediate progenitor cells and are positive for distal-less homeobox 2 (Dlx2) and achaete-scute complex homolog-1 (Mash1) (Lois and Alvarez-Buylla, 1994; Doetsch et al., 1999; Carleton et al., 2003; Ma et al., 2009). In rodent brains, C-cells produce neuroblasts (A-cells) which migrate along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate to interneurons (Lois and Alvarez-Buylla, 1994; Belluzzi et al., 2003; Carleton et al., 2003) whereas in human brains neuroblasts populate the striatum (Bergmann et al., 2012; Ernst et al., 2014).

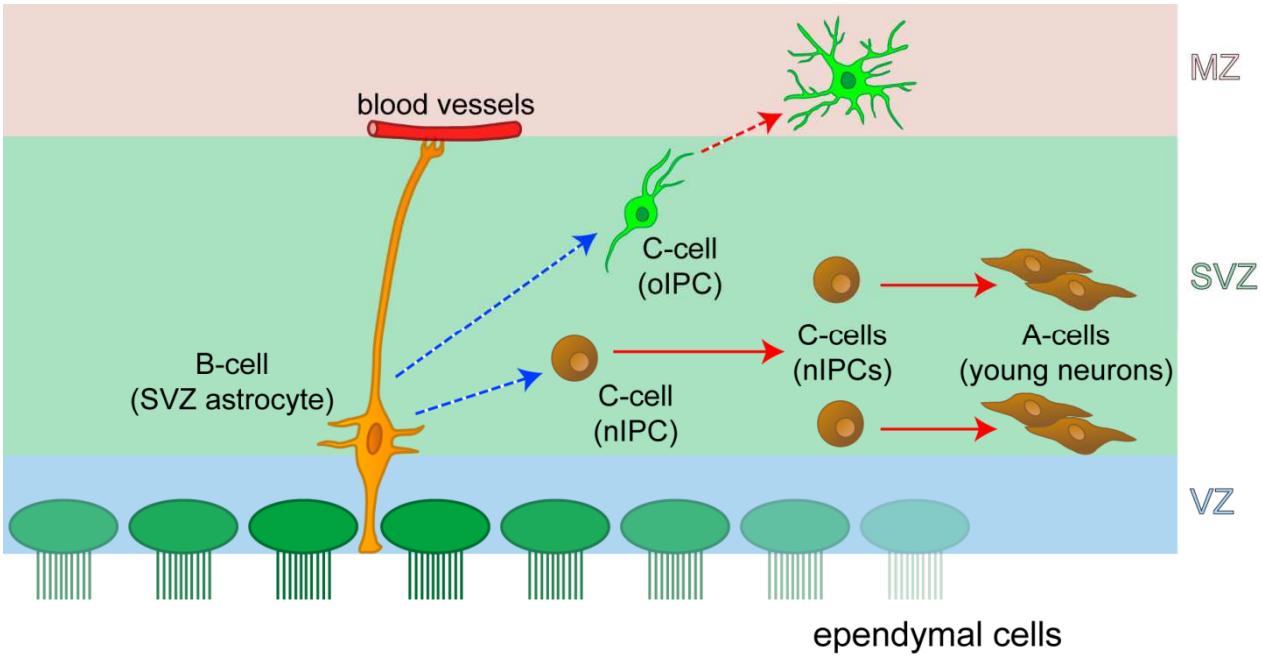

Figure 2.4: Neural progenitor cells in the adult neural stem cell niche give rise to neurons or oligodendrocytes

Radial glial cells develop into SVZ astrocytes (B-cells), which function as neural stem cells in the adult brain. On the apical site, B-cells contact the ventricle and at the basal site blood vessels. B-cells are able to give rise to C-cells known as transit-amplifying progenitors by asymmetric cell division which are intermediate progenitor cells and have the potential to generate neuroblasts (type A-cells). In the rodent brain, A-cells migrate along the RMS to the olfactory bulb and differentiate into interneurons. Furthermore, the expression of transcription factors such as Olig2 can induce an oligodendroglial differentiation of B- and, in lower numbers, also of C-cells. MA: mantle; nIPCs: neurogenic progenitor cell; NSCs: neural stem cells; oIPCs: oligodendrocytic progenitor cell, SVZ: subventricular zone; VZ: ventricular zone. Adapted from Kriegstein and Alvarez-Buylla, 2009.

Besides differentiation into neurons, B-cells can give rise to glial cells depending on the transcriptional cues (Menn et al., 2006; Benner et al., 2013). For example, the

expression of the oligodendrocyte transcription factor 2 (Olig2) in B-cells and in some transit-amplifying progenitors leads to a non-neuronal cell fate toward oligodendrocytes which migrate from the SVZ to the corpus callosum, striatum and fimbria fornix (Fogarty et al., 2005; Hack et al., 2005; Casper and McCarthy, 2006; Menn et al., 2006; Seri et al., 2006) where they differentiate to neural/glial antigen 2 (NG2)-positive and mature myelinating oligodendrocytes (Menn et al., 2006).

2.1.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent adult stem cells originating from the mesoderm (Friedenstein et al., 1966; Friedenstein et al., 1970; Friedenstein et al., 1974; Friedenstein, 1976; Friedenstein et al., 1976; Friedenstein et al., 1978). MSCs are also referred to as multipotent stromal cells or mesenchymal stromal cells which have the potential to differentiate into several cell types such as osteoblasts, chondrocytes, adipocytes, fibroblasts and myoblasts (Friedenstein et al., 1966; Friedenstein et al., 1970; Friedenstein et al., 1974; Friedenstein, 1976; Friedenstein et al., 1976; Friedenstein et al., 1978; Bruder et al., 1997; Kadiyala et al., 1997; Ferrari et al., 1998; Young et al., 1998; Dennis et al., 1999). While initially identified and isolated from bone marrow, MSCs can also be isolated from many other tissues such as amniotic fluid, dental tissue, adipose tissue or umbilical cord blood (da Silva Meirelles et al., 2006; Gazit Z., 2019). Although MSCs have multipotent properties, the differentiation potential differs depending on their origin. This is reflected by the observation that umbilical cord blood-derived MSCs are also able to differentiate into endothelial cells, a lineage that cannot be generated by bone marrow-derived MSC (Wu et al., 2007).

Cultured MSCs have to meet a minimal set of standard criteria determined by the International Society of Cell Therapy (ISCT) (Dominici et al., 2006). According to these criteria, MSCs are defined by the potential to differentiate into osteoblasts, adipocytes and chondrocytes, adherence to plastic and high expression of CD105, CD73 and CD90 as well as low expression of CD45, CD34, CD14, CD11b, CH79alpha, CD19 and HLA-DR surface molecules (Figure 2.5) (Dominici et al., 2006; Naji et al., 2019).

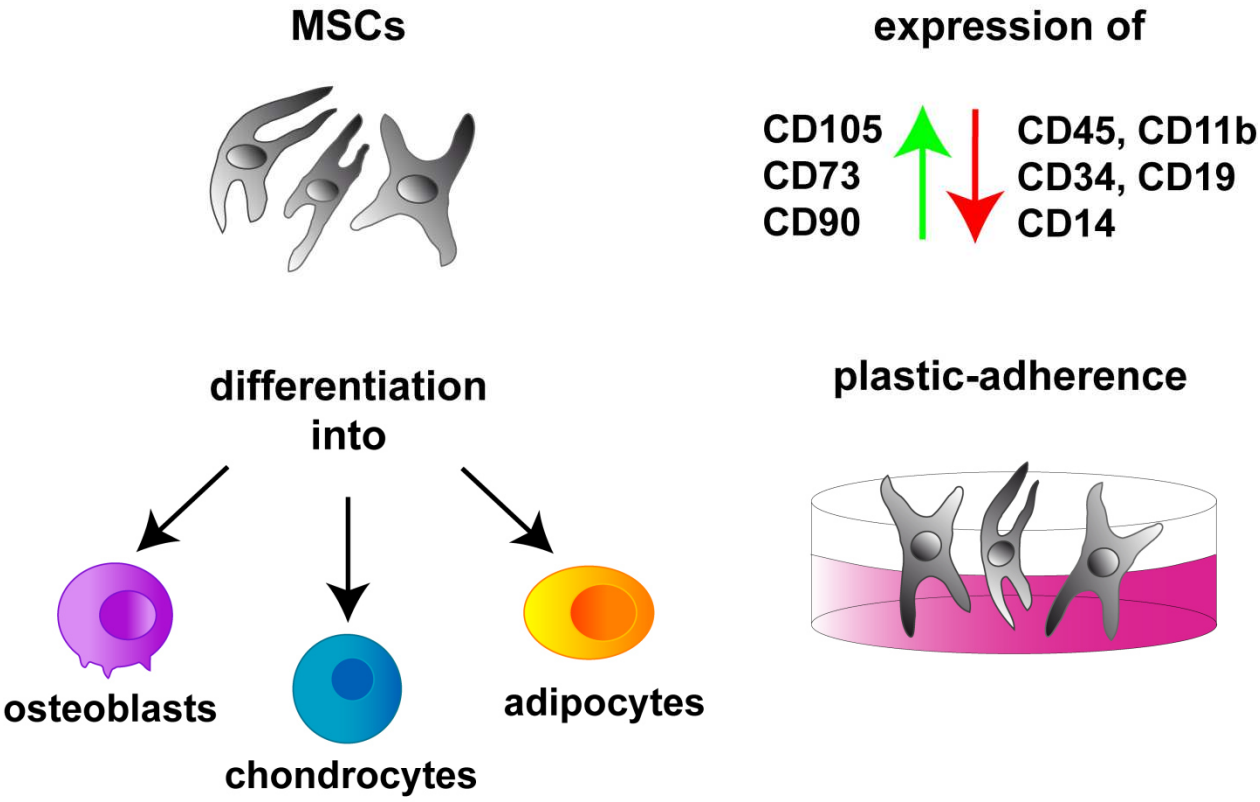

Figure 2.5: Characterization of mesenchymal stem cells

Mesenchymal stem cells are defined by a minimal of set of standard criteria established by the International Society of Cell Therapy (ISCT) including the potential to differentiate into osteoblast, chondrocytes and adipocytes. Furthermore, MSCs express specific surface marker combination and adhere to plastic.

MSCs have a great potential for clinical therapies due to their positive effects in tissue repair and regeneration, providing anti-inflammatory factors (Le Blanc et al., 2008; Jeong et al., 2014), however, MSCs show a limited capacity to differentiate and proliferate due to senescence and the donor age (Hayflick, 1965; Mets and Verdonk, 1981; Stenderup et al., 2003; Bonab et al., 2006; Mareschi et al., 2006; Noer et al., 2007; Rivera et al., 2019).

2.1.2.1 Mesenchymal stem cell-secreted factors

One important feature of MSCs is the secretion of trophic factors influencing the environment and homeostasis (Maitra et al., 2004; Caplan, 2007; Jadasz et al., 2018; Naji et al., 2019; Samper Agrelo et al., 2020). For example, MSC secrete the neurotrophic factors glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) as well as insulin-like growth factor 1 (IGF-1) and stromal cell-derived factor 1 (SDF-1; CXCL12) known to influence neurogenesis (Zhang et al., 2004; Horita et al., 2006; Lin et al., 2011; Jeong et al., 2014) as well as

angiopoietin 1 (Ang-1), erythropoietin (EPO) and vascular endothelial growth factor (VEGF) regulating angiogenesis (Zwezdaryk et al., 2007; Beckermann et al., 2008; Mishra et al., 2008; Pedersen et al., 2014). Furthermore, MSCs have immunosuppressive properties mediated by secretion interleukins, e.g., interleukin-6 (IL6) and IL10, transforming growth factor β (TGF-β) or indoleamine 2,3-dioxygenase (IDO) and the vascular cell adhesion molecule 1 (VCAM-1/CD106) (Beyth et al., 2005; Najar et al., 2009; Ren et al., 2009; Patel et al., 2010; Yang et al., 2013). Due to the secretion of various factors, MSCs exert a high regenerative potential and are likely suitable for cell transplantations or tissue engineering approaches (Le Blanc et al., 2008; Kordelas et al., 2014).

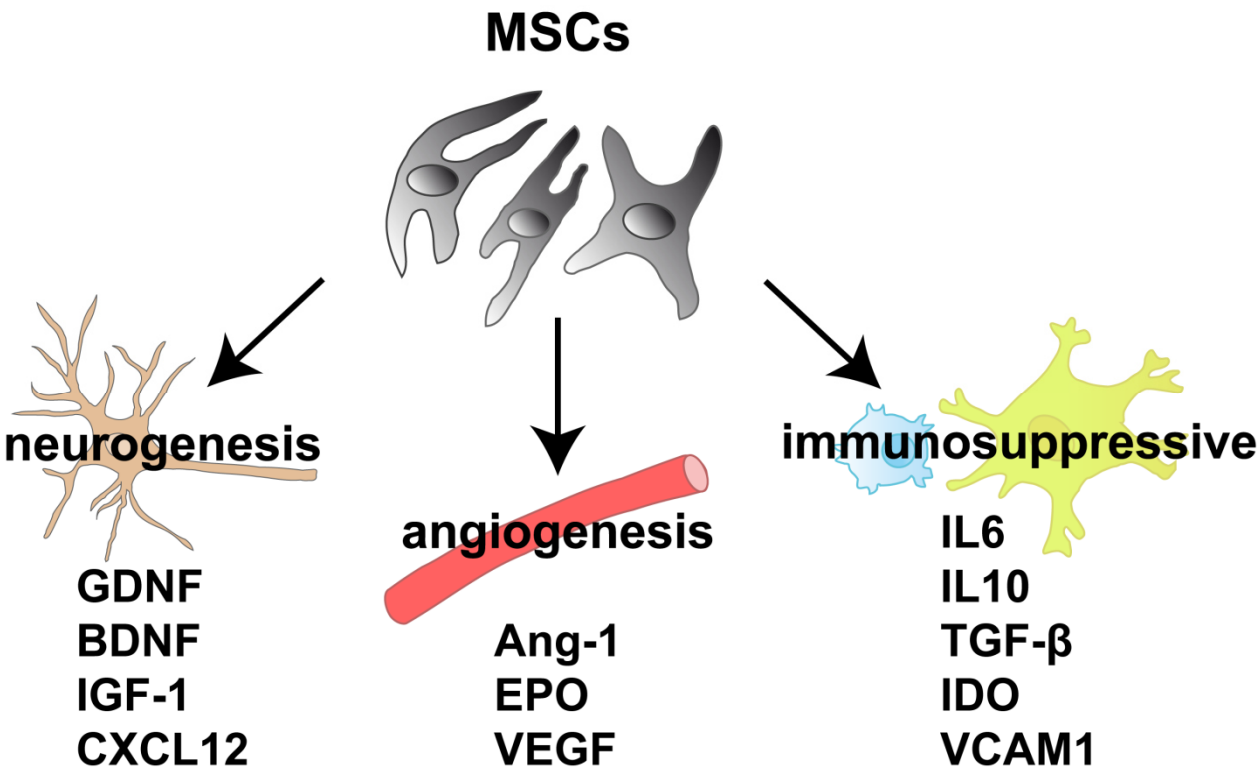

Figure 2.6: MSC-secreted factors interact with the environment

By secretion of various chemokines, growth factors and cytokines MSCs influence, e.g., angiogenesis and the immune reaction. Furthermore, MSCs secrete factors known to influence neurogenesis, e.g., after transplantation into injured CNS tissue.

To identify secreted proteins, mass spectrometry based secretome analysis was performed revealing regenerative, anti-inflammatory or tumor-promoting properties of MSCs (Maffioli et al., 2017; Spitzhorn et al., 2017; Teixeira et al., 2017; Baberg et al., 2019; Jungbluth et al., 2019; Timaner et al., 2020). For example, Maffioli and colleagues showed that stimulation with pro-inflammatory cytokines (IL1b, IL6 and

tumor necrosis factor α [TNFα]) led to an enrichment of MSC-derived proteins known to be involved in inflammatory processes and angiogenesis. Furthermore, the tissue inhibitor of metalloproteinase-1 (TIMP-1) as a key effector molecule responsible for anti-angiogenic properties was identified (Maffioli et al., 2017). Another secretome analysis of amniotic fluid-derived MSCs showed that cytokines such as CC-chemokine ligand 2 (CCL2), CXCL 1, IL6, and IL8 were highly abundant whereas CXCL12 and IL21 were low abundant (Spitzhorn et al., 2017). iPSC-derived MSCs secrete proteins with immunomodulatory- as well as osteo-regenerative properties including platelet-derived growth factor (PDGF)-AA, osteopontin (OPN), serpin family member 1 (serpin E1) and angiogenin (Ang) (Jungbluth et al., 2019). Furthermore, MSCs secrete proteins with known neuroregulatory functions, e.g. cystatin C (CysC; Cst3), glia-derived nexin (GDN), galectin-1 (Gal-1), pigment epithelium-derived factor (PEDF), VEGF, brain-derived neurotrophic factor (BDNF), IL6, and GDNF which were identified using a combined mass spectrometry analysis and bioplex assay (Teixeira et al., 2017). A combinatory secretome and proteome analysis revealed the secretion of cytokines and growth factors which are involved in the physiological regulation of hematopoiesis. Here, VEGF-C, TGF-β1, TGF-β2 and growth differentiation factor (GDF) 6 were detected (Baberg et al., 2019).

While secretion through the endoplasmic reticulum (ER)-Golgi route displays classical protein secretion, also unconventional secretion routes exist (Rabouille et al., 2012; Steringer et al., 2014; Pompa et al., 2017). Here, proteins do not pass the ER and Golgi apparatus but are taken up by, for example, exosomes and transported to the membrane (Pompa et al., 2017). Furthermore, fibroblast growth factor (FGF) 2 passes an alternative secretion pathway characterized by a direct translocation across the plasma membrane (Steringer et al., 2014).

2.1.2.2 Mesenchymal stem cells and their impact on neural stem cells

While nearly all studies addressed the immunomodulating and regenerative properties of MSCs, little is known about the influence of MSCs and their secreted factors on glial cells, especially on oligodendroglia. River and colleagues showed that MSCs induce an oligodendroglial fate of aNSCs at an expense of astrogenesis as presumed by the authors via the secretion of MSC-derived factors (Rivera et al., 2006). Beside cell-cell contact, paracrine signaling by MSC-derived secreted factors were shown to promote an oligodendroglial differentiation despite an astroglial

environment (Steffenhagen et al., 2012). Beside NSCs also OPCs differentiation can be promoted by MSCs and their secreted factors (Jadasz et al., 2013). Furthermore, rat as well as human MSC-secreted proteins were shown to promote an oligodendroglial differentiation of rat NSCs as well as human iPSCs-derived NSCs (Jadasz et al., 2018). The strong impact of MSC-secreted factors was also shown in the inflammatory mediated experimental allergic encephalitis (EAE) model which leads to demyelination in the CNS. Here, treatment with human MSC-conditioned medium (MSC-CM) led to functional repair by promoting the development of oligodendrocytes and neurons (Bai et al., 2012).

2.1.3 Induced pluripotent stem cells

In 2006, iPSCs were generated from mouse fibroblasts (embryonic and adult) (Takahashi and Yamanaka, 2006) by retrovirus induced ectopic expression of the transcriptional factors octamer binding transcription factor 4 (Oct4), Sox2, krueppel-like factor 4 (Klf4) and v-myc myelocytomatosis viral oncogene homolog (c-myc) (Takahashi et al., 2007a). Similar to ESCs, iPSCs express most of the ESCs marker proteins such as Nanog, embryonic stem cell-expressed Ras (ERas), zinc finger protein 42 (zfp42) and undifferentiated embryonic cell transcription factor 1 (Utf1) and exhibit identical morphological features (Figure 2.7) (Takahashi et al., 2007a). Beside fibroblasts, iPSCs can be generated from several other cell types like liver or stomach cells (Aoi et al., 2008), amniotic fluid cells (Wolfrum et al., 2010), blood cells (Chou et al., 2011), MSCs (Megges et al., 2015) or urine cells (Bohndorf et al., 2017). Reprogramming efficiency was further optimized by using single polycistronic vectors for integration into the genome (Carey et al., 2009) and by using non-integrating adenoviruses to sustain an unmodified genome (Zhou and Freed, 2009). Furthermore, small molecules were used for reprogramming as well as mini-circle DNA instead of viruses (Huangfu et al., 2008; Shi et al., 2008; Yu et al., 2011; Drews et al., 2012; Zhu et al., 2014).

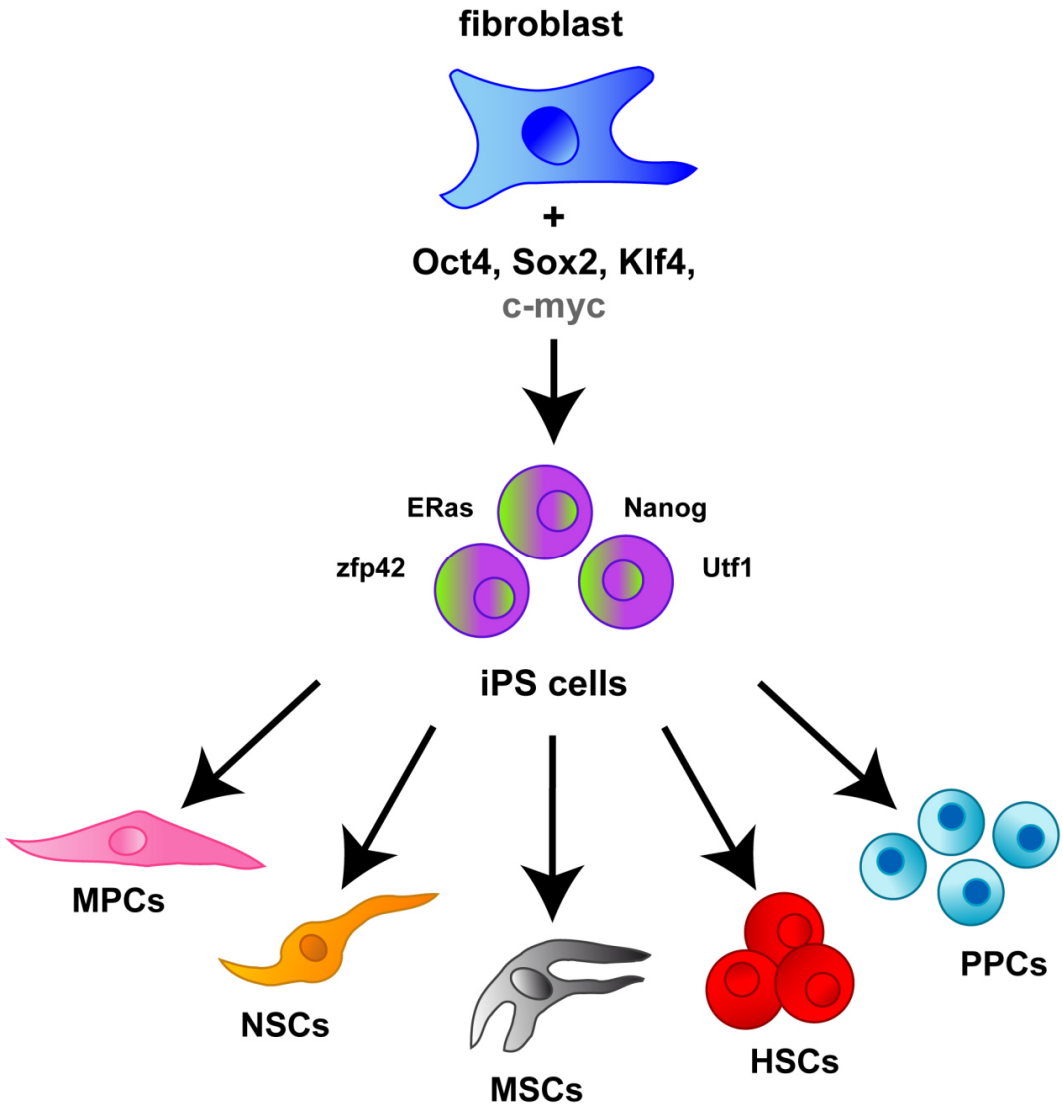

Figure 2. 7: Generation and differentiation of induced pluripotent stem cells

Among other cell types fibroblasts can be reprogrammed to iPSCs by retroviral transduction inducing an ectopic expression of the transcription factors Oct4, Sox2, Klf4 and c-myc. iPSCs express several ESC markers such ERas, Nanog, zfp42 and Utf1, thus representing a substitute for ESCs. iPSCs can be further used to generate almost all cell types of the three germ layers like MSCs, NSCs, HSCs, pancreatic progenitor cells (PPCs) or muscle progenitor cells (MPCs).

iPSCs are increasingly used to study disorders such as Alzheimer's disease, Parkinson's disease or hepatitis (Israel et al., 2012; Miller et al., 2013; Schöbel et al., 2018). Future perspective could be that patient derived cell types are reprogrammed and transplanted into the diseased tissue (Mandai et al., 2017; Spitzhorn et al., 2018; Jungbluth et al., 2019). Interestingly, several studies using animal disease models showed that iPSC-derived MSCs are able to participate in nervous system regenerative processes (Wang et al., 2014; Hawkins et al., 2018; Spitzhorn et al., 2018; Jungbluth et al., 2019). iPSCs can be differentiated into neural precursor cells

(NPCs) and OPCs differentiating into myelinating oligodendrocytes (Czepiel et al., 2011). Furthermore, in a viral model of multiple sclerosis (MS) human iPSC derived NPCs promote recovery (Chen et al., 2014) and showed neuroprotective properties after transplantation into an EAE model due to the secretion of leukemia inhibitory factor (LIF) (Laterza et al., 2013).

2.2 Oligodendroglial cells

Oligodendrocytes are the myelinating cells of the CNS which are essential for correct brain function by providing electrical isolation of axons, accelerated signal propagation and axon nutrition (Fünfschilling et al., 2012; Saab et al., 2016). Oligodendroglial development starts during embryonic and is maintained up to postnatal stages (Pringle and Richardson, 1993; Timsit et al., 1995; Spassky et al., 1998; Tekki-Kessaris et al., 2001; Kessaris et al., 2006). During telencephalon development, OPCs develop in different phases. In the first phase, early oligodendrocytes develop around mouse embryonic day E12.5 from homeobox protein Nkx2.1-expressing progenitor cells localized in the ventral telencephalon (Spassky et al., 1998; Woodruff et al., 2001; Kessaris et al., 2006). In a second phase, GS homeobox 2 (Gsx2)-positive oligodendroglial cells develop within the lateral/caudal ganglionic eminence whereas Emx1-positive cortical progenitor cells in the dorsal SVZ give rise to oligodendroglial cells in a third phase. Of note, most of these early Nkx2.1-oligodendrocytes are eliminated in early adulthood, thus the majority of oligodendrocytes and OPCs persisting in the adult cortex have an Emx-1 expressing origin (Kessaris et al., 2006; Rowitch and Kriegstein, 2010). In the adult CNS, oligodendrocytes can also be generated from resident OPCs. These precursor cells derived from neuroepithelial and radial glia cells of the VZ and SVZ during telencephalon development, are widely distributed throughout the adult CNS and are implicated in natural cell turnover or replacement in disease or following injury (Chang et al., 2000; Kessaris et al., 2006; Kriegstein and Alvarez-Buylla, 2009).

OPCs are positive for Olig2 and Sox10 as well as NG2 and platelet-derived growth factor receptor alpha (PDGFRα). While Olig2 and Sox10 are constitutively expressed through the complete oligodendroglial lineage, OPCs lose the expression of NG2 and PDGFRα during maturation towards oligodendrocytes (Richardson et al., 1988; Pringle and Richardson, 1993; Nishiyama et al., 1996; Nishiyama et al., 1999). At this developmental stage, OPCs are able to migrate and proliferate (Barateiro and

Fernandes, 2014). During maturation, both in development as well as in the adult CNS, OPCs give rise to pre-oligodendrocytes which express O-antigen 4 (O4) and G protein-coupled receptor 17 (GPR17) (Sommer and Schachner, 1981; Boda et al., 2011; Barateiro and Fernandes, 2014). Pre-oligodendrocytes further develop into immature oligodendrocytes characterized by the downregulation of OPC-markers and upregulation of galactocerebroside C (GalC) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) (Sprinkle, 1989; Yu et al., 1994). Immature oligodendrocytes further differentiate to mature oligodendrocytes expressing myelin associated proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG) and proteolipid protein (PLP) (Macklin et al., 1987; Brunner et al., 1989; Scolding et al., 1989; Huang et al., 2012; Barateiro and Fernandes, 2014) whereof MBP and PLP are the main myelin proteins (Folch and Lees, 1951; Zeller et al., 1984; de Ferra et al., 1985). The specific combination of myelin proteins is essential for the myelin sheaths formation. As soon as oligodendrocytes get contact to axons, their processes expand and wrap around the axon in concentric membrane layers resulting in myelin sheaths (Sobottka et al., 2011). Changes in the marker expression pattern reflecting the different developmental stages are accompanied by morphological changes. OPCs are characterized by a small polygonal soma with fine processes when localized in grey matter (Kukley et al., 2010) whereas in the white matter OPCs have a few short processes and a bipolar morphology (Chittajallu et al., 2004). Pre-oligodendrocytes display multipolar short processes whereas immature oligodendrocytes have long ramified branches and mature oligodendrocytes develop strong elongated processes which form myelinated sheaths (Figure 2.8) (Barateiro and Fernandes, 2014).

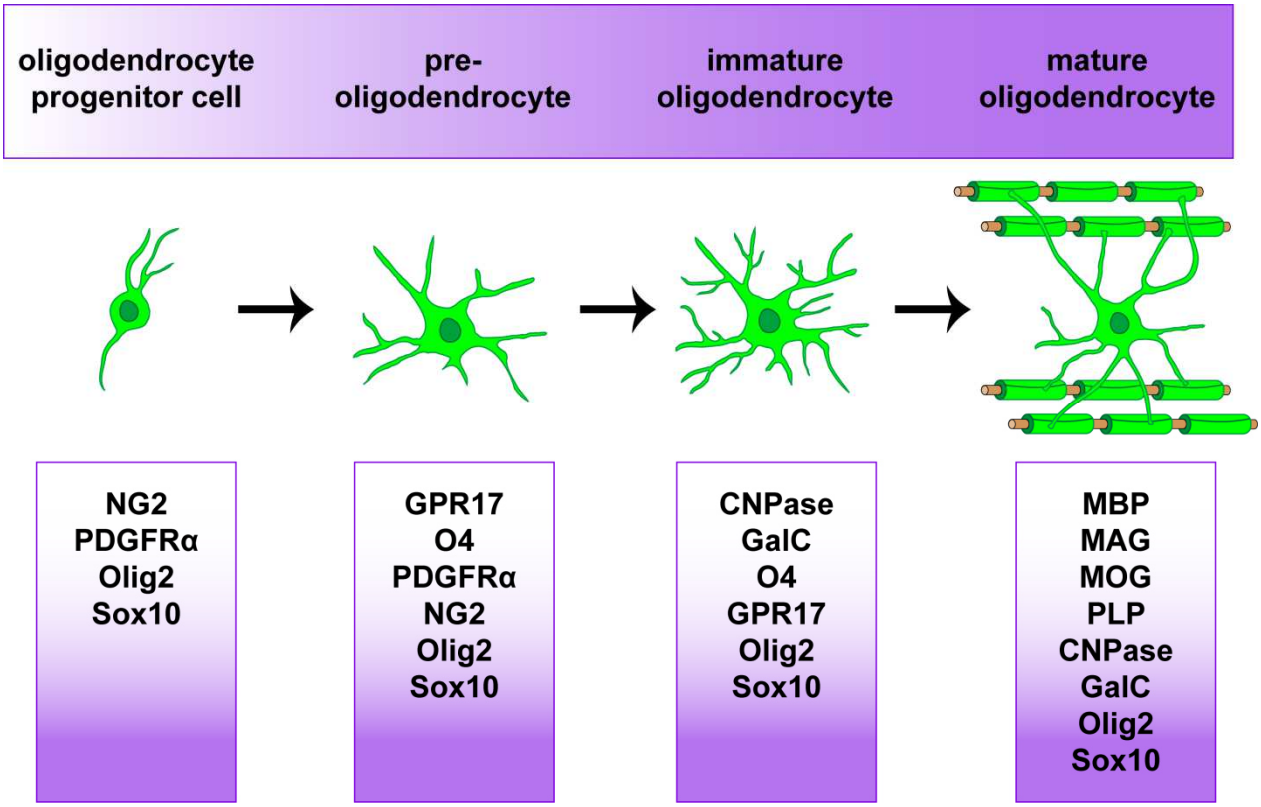

Figure 2.8: Stage-specific marker expression and morphological development during oligodendroglial lineage differentiation

OPCs differentiate from pre-oligodendrocytes and immature oligodendrocytes into mature myelinating oligodendrocytes. These stages differ from each other by their morphology, marker expression as well as the potential to proliferate and migrate. CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; GalC, galactocerebroside C; GPR17, G protein-coupled receptor 17; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; O4, O-antigen 4; Olig1/2, oligodendrocyte transcription factor 2; PDGFRα, platelet-derived growth factor; PLP, proteolipid protein; Sox10, sex determining region Y-Box transcription factor 10.

Oligodendrocytes extend numerous processes to form concentric layers of membranes containing myelin and internode segments laterally along axons. This process is regulated by several factors such as the appearance of the myelin markers MBP, MOG, MAG and PLP, the changes in cell morphology, regulation of myelin genes through neurons via the expression and secretion of growth factors, neurotrophic factors and their electrical activity (Demerens et al., 1996; Barres and Raff, 1999; Snaidero et al., 2014; Hughes et al., 2018). This myelination process starts around postnatal stage P2 with an extensive myelination up to P14 (Hartman et al., 1979; Bjelke and Seiger, 1989; Hardy and Friedrich, 1996; Dean et al., 2011). Normally, myelination begins in the caudal part of the brainstem region and continues up to the forebrain thus proceeding from posterior to rostral (Coffey and McDermott, 1997). Myelin sheath formation is regulated by extrinsic signals as well as by intrinsic

regulatory cues (Emery, 2010). Apart from transcription factors that control the differentiation of OPCs into mature oligodendrocytes and thereby myelination, other factors that can regulate myelination such as myelin gene regulatory factor (MRF) which is required for the expression of myelin genes (Emery et al., 2009) or the mammalian target of rapamycin (mTOR) and particularly mTOR complex 1 (mTORC1) are key regulators of oligodendrocyte maturation and myelination (Lebrun-Julien et al., 2014; McLane et al., 2017). Extrinsic regulatory factors, e.g. PDGF-α which is expressed by neurons as well as astrocytes regulates survival and proliferation of OPCs (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Emery, 2010). Neuron-derived molecules such as neuregulin (Fernandez et al., 2000) or also negative regulators such as polysialic acid neural cell adhesion molecule (PSA-NCAM) (Charles et al., 2000) and leucine rich repeat and immunoglobin-like domain-containing protein 1 (LINGO-1) (Mi et al., 2005; Mi et al., 2013) regulate oligodendroglial maturation and myelination. Another negative regulator of the oligodendroglial maturation is the cyclin-dependent kinase inhibitor p57kip2 (Jadasz et al., 2012; Göttle et al., 2015). The subcellular distribution of p57kip2 changes during differentiation of oligodendroglial cells resulting exclusion of the protein from the nucleus which results in an enhanced myelin protein expression, more complex morphological phenotype and enhanced myelination *in vitro* (Göttle et al., 2015; Jadasz et al., 2018). In addition, neuronal activity has an impact on myelination of the CNS (Gibson et al., 2014; Hines et al., 2015) as well as on OPC proliferation, differentiation and survival (Barres and Raff, 1993; Hughes et al., 2018). OPCs get synaptic input which induces myelination (Gibson et al., 2014; Hines et al., 2015; Mensch et al., 2015) through neuronal derived the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Bergles et al., 2000) and gamma-aminobutyric acid (GABA) (Lin and Bergles, 2004) receptors. It was further shown that the axon-glia interaction and the domain organization of myelinated axons require neurexinIV/Capsr/paranodin (Peles and Salzer, 2000; Bhat et al., 2001).

Furthermore, it is known that white matter (WM) OPCs differentiate more frequently into mature myelinating oligodendrocytes as compared to OPCs derived from and/or residing in grey matter (GM) (Dimou et al., 2008) and that the WM in general appears to be more supportive for oligodendrocyte differentiation (Vigano et al., 2013; Yeung et al., 2014). These differences may be due to a lower OPC density and slower

proliferation rate of GM OPCs which results in less mature oligodendrocytes (Dimou et al., 2008; Rivers et al., 2008; Kang et al., 2010; Zhu et al., 2011).

2.2.1 NSC differentiation into mature oligodendrocytes

Besides OPCs, oligodendrocytes can also be generated from adult SVZ NSCs (B-cells) (Menn et al., 2006; Nait-Oumesmar et al., 2008; Xing et al., 2014; Chavali et al., 2018; van Tilborg et al., 2018), particularly under pathological conditions. Stem cell mediated oligodendrogenesis was shown to be regulated by different transcriptional cues such Olig1/2, Sox10 and Nkx2.2 (Hack et al., 2005; Pozniak et al., 2010; Tong and Alvarez-Buylla, 2014; van Tilborg et al., 2018). Furthermore, the transcription factor achaete-scute homolog 1 (Ascl1/Mash1), which normally plays a role in neurogenesis, was also shown to promote brain oligodendrogenesis during myelination and remyelination processes (Nakatani et al., 2013). NSCs differentiate into remyelinating oligodendrocytes after overexpression of the transcription factor Zfp 488 (Soundarapandian et al., 2011). In addition, growth factors such as IGF-1, thyroid hormone T3, cytokine ciliary neurotrophic factor (CNTF), PDGF, EGF and members of the FGF family are known to promote and oligodendroglial differentiation (Armstrong et al., 2002; Woodruff et al., 2004; Murtie et al., 2005; Aguirre et al., 2007; Vana et al., 2007; Gonzalez-Perez et al., 2009; Miron et al., 2011; Vernerey et al., 2013). Furthermore, bone morphogenic protein (BMP) signaling was shown regulate oligodendroglial differentiation by upregulation of chordin, a BMP antagonist, in the SVZ resulting in new oligodendrocytes in the corpus callosum after demyelination (Jablonska et al., 2010). In addition, intraventricular infusion of Noggin in a cuprizone-induced demyelination model increases the number of oligodendrocytes in the SVZ (Cate et al., 2010). The canonical Wnt signaling pathway is also known to promote oligodendrogenesis by the activation of quiescent aNSCs in the SVZ and further by the inhibition of glycogen synthase kinase 3β (GSK3β) which induces oligodendrogenesis in the dorsal SVZ during development and adulthood (Ortega et al., 2013; Azim et al., 2014a; Azim et al., 2014b). It was further shown that during a demyelination, SVZ-derived NSCs are activated which depends on the downregulation of active Cdc42 levels which in turn downregulates Notch signaling and allows for an upregulation of Wnt/β-catenin targets (Chavali et al., 2018). Another protein with pro-oligodendroglial lineage properties is the enhancer of zeste homolog 2 (Ezh2) which is a polycomb group protein involved in stem cell renewal and

maintenance by inducing gene silencing via histone methylation and deacetylation. Ezh2 was found to be highly expressed in NSCs that differentiate into an oligodendrocytic cell lineage and an increase of Ezh2 also results in an increase of oligodendroglial lineage cells (Sher et al., 2008). Beside of the positive regulators also negative regulators control the differentiation of NSCs towards an oligodendroglial lineage. The extracellular matrix molecule tenascin C acts on the OPC maintenance and reduces the MBP expression in oligodendrocytes (Czopka et al., 2010). Furthermore, the deletion of neurfibromin 1 (Nf1) was shown to conduct ectopic oligodendrogenesis from hippocampal subgranular zone (SGZ)-derived NSCs (Sun et al., 2015). The inhibitor p57kip2 is also known to influence the NSC-oligodendrogenesis depending on its intracellular localization (Jadasz et al., 2012; Göttle et al., 2015; Jadasz et al., 2018). Transplantation of p57kip2 suppressed SGZ- and SVZ-derived NSCs into the intact as well as injured dorsal rat spinal cord revealed an enhanced oligodendroglial differentiation of NSC (Jadasz et al., 2012; Beyer et al., 2020).

While the above mentioned transcription and growth factors are known to be important for NSCs as well as OPCs oligodendrogenesis, some factors are unique for NSCs regulation. For example, inactivation of sirtuin-1 (Sirt1), normally implicated in energy metabolism, increases NSC-derived oligodendrocyte differentiation (Rafalski et al., 2013). Moreover, NSCs are recruited to lesion sites differentiating into oligodendrocytes exclusively due to inhibition of Gli1, a downstream mediator of Shh signaling (Samanta et al., 2015). In addition, depletion of Drosha enables ectopic oligodendrogenesis via recruitment of nuclear factor IB (NFIB) that initiates pro-oligodendroglial transcriptional programs in SGZ-derived NSCs (Rolando et al., 2016). Similarly, depletion of the transcription factor NFIX or of B-cell leukemia homeodomain 1 (Pbx1) results in an increased oligodendrogenesis of SVZ-NSCs and TAPs (Zhou et al., 2015; Grebbin et al., 2016). Beside the negative factors, the transcription factor prospero-related homeobox 1 gene (Prox1) known to be important for neurogenesis in the SGZ, was shown to promote NSC-oligodendrogenesis in the SVZ (Bunk et al., 2016).

2.3. Myelin

In the CNS, oligodendrocytes ensheath axons via the generation of myelin and the extension of their processes during differentiation (Bunge et al., 1962; Demerens et al., 1996; Barres and Raff, 1999; Baumann and Pham-Dinh, 2001; Snaidero et al.,

2014; Hughes et al., 2018). The developmental myelination process was described as simultaneous radial and longitudinal expansion of the upcoming myelin sheaths by adding new membrane at the growing tip of the inner tongue (Snaidero et al., 2014). At the same time, the process commonly referred as wrapping takes place as the innermost layer of the sheath expands laterally and presses itself between the preceding layer and the axon (Nave and Werner, 2014; White and Kramer-Albers, 2014). Myelin has a high lipid content (around 70-75%) which includes cholesterol, phospholipids, galactolipids and plasmalogens (Norton and Poduslo, 1973; Saher et al., 2005). Due to the high lateral mobility of myelin lipids, the cholesterol content is likely responsible to limit the membrane's fluidity, thereby affecting intracellular transport and myelin compartmentalization (Gould and Dawson, 1976; Rosetti et al., 2008; Maxfield and van Meer, 2010; Yurlova et al., 2011). Myelin in the CNS can be divided into compact and non-compact myelin (Bunge et al., 1962; Baumann and Pham-Dinh, 2001; Nave and Werner, 2014). The compaction of multiple membrane layers occurs by a growing sheath expanding underneath the previous layer which is a result of the compaction progression of the abaxonal layers toward the (adaxonal) inner tongue with a delay of two to three wraps behind the leading edge (Hildebrand et al., 1993; Snaidero et al., 2014). This process necessitates specialized adhesive proteins as well as the removal of molecules that prevent compaction (Musse et al., 2008; Nawaz et al., 2009; Nawaz et al., 2013; Nave and Werner, 2014). Due to highly charged phospholipids such as PIP_2, the intracellular surface of the glial membrane is initially inhibitory to tight appositions. Nevertheless, basic proteins such as MBP, which displays a high affinity to PIP_2, gets attracted caused by a negatively charged membrane (Musse et al., 2008; Harauz et al., 2009; Nawaz et al., 2009; Nawaz et al., 2013). Through the neutralization of membrane phospholipids, MBP pulls two bilayers together representing the major dense line and is responsible for the myelin grow (Min et al., 2009; Aggarwal et al., 2013). Adhesive forces between neighboring myelin layers approach at the radial component, which is primarily composed of claudin-11 (Cldn11), thus serial tight junction strands undulating through compact central myelin (Peters, 1961; Gow et al., 1999; Rosenbluth et al., 2006; Devaux and Gow, 2008; Rosenbluth et al., 2009; Nave and Werner, 2014). Beside of the compacted myelin layer there is also existing a non-compacted myelin layer. Paranodal channels, which remain non-compacted throughout life, provide cytoplasmic continuity between the glial cell body and the axonal non-compacted

myelin layer (Arroyo and Scherer, 2000; Velumian et al., 2011; Nave and Werner, 2014). Additional cytoplasmic channels remain open during early CNS myelination but are largely closed during the maturation of CNS myelin (Arroyo and Scherer, 2000; Velumian et al., 2011). These radial cytoplasmic channels provide routes for vesicular trafficking toward the growth zone at the inner tongue during early CNS myelination (Snaidero et al., 2014). Here, absence of CNPase speeds up the MBP-mediates developmental closure of these incisures but it still remains unclear if a normal channel closure implies CNPase's microtubule-modifying or enzymatic activities or if CNPase is just difficult to displace due to its wealth size or ability to dimerize (Lee et al., 2005; Myllykoski et al., 2012; Verrier et al., 2013; Nave and Werner, 2014). In general, during compaction, molecules with cytoplasmic domains larger than those of a certain threshold size do not enter mature myelin (Aggarwal et al., 2011). Furthermore, inhibitory to efficient myelination is the forced attachment of a polysialic acid extracellular moiety to proteins normally incorporated into the growing sheath, hence glycosylated proteins are generally absent from compact CNS myelin (Fewou et al., 2007; Bakhti et al., 2013; Nave and Werner, 2014).

2.3.1 Demyelination and remyelination

Apart from the naturally occurring cell turnover, oligodendroglial cell replacement is necessary in neurological diseases, traumata, ischemia and autoimmune attacks during which oligodendrocytes are destroyed and axons demyelinated (Kuhn et al., 2019). While after traumatic injuries or ischemia oligodendrocyte loss and demyelination occur in a secondary process, oligodendrocytes are direct targets in several autoimmune diseases such as multiple sclerosis (MS) (Franklin, 2002; Kuhlmann et al., 2008; Kremer et al., 2011). In the CNS, MS is the most common autoimmune disease (Love, 2006) characterized by a T cell mediated autoimmune attack against myelin components (Weiner, 2004; Saxena et al., 2008). T cells secrete several factors, e.g., interferon-γ (IFN-γ), IL17A, IL17F, IL22, IL4, IL10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) which activates astrocytes, microglia, but also lymphocytes which in turn secrete a large number of inflammatory chemokines and cytokines thereby recruiting peripheral immune cells to sites of inflammation (Kurschus, 2015; Zhang et al., 2018b). Activated microglia secrete CC-chemokine ligand (CCL) 2, CCL18, CCL22, CXCL13, CXCL12 and CX3CL1 (Kohler et al., 2008; Moriguchi et al., 2013; Blauth et al., 2015; Janssen et

al., 2016; Hendrickx et al., 2017; Londoño and Mora, 2018; Cui et al., 2020) and TNF-*α*, IL-6, IL-12, IL-4, IL-10 and TGF-*β* (Luo et al., 2017) and can damage myelin sheaths and oligodendrocytes (Prinz and Priller, 2014). Microglia, macrophages and lymphocytes attack oligodendrocytes thus leading to demyelination and consequently axonal degeneration resulting in several neurological symptoms (Sospedra and Martin, 2005; Blackmore and Letourneau, 2006; Dhib-Jalbut, 2007; Lassmann et al., 2007; Trapp and Nave, 2008).

The disease progression of MS is variable, however, three major clinical forms are described: primary progressive (PP), secondary progressive (SP) and relapsing-remitting (RR) MS (Lublin and Reingold, 1996). While PP (10% of MS diagnoses) is characterized by a permanent aggravation of symptoms without remission periods, patients suffering from RR undergo episodes of relapse meaning functional deficits due to inflammatory caused demyelination followed by a period of functional recovery (remission) (Lublin and Reingold, 1996). Of note, approximately half of the RR patient also suffer from SP in which symptoms become more pronounced (Lublin and Reingold, 1996).

Due to the complex nature of the disease, many environmental, genetic and viral factors have an influence on the first occurrence of MS (Willer and Ebers, 2000). Studies have shown that the risk of developing MS is higher for family relatives of patients with diagnosed MS (Sadovnick et al., 1996). Furthermore, the human leukocyte antigen (HLA) genes HLA-DR1501 and HLA-DQ0601 resulted in a higher risk of developing MS (Compston et al., 1994). Another genetic alteration, the combination of the plasminogen activator inhibitor-1 (PAI-1) 5G/5G genotype and tissue-type plasminogen activator (TPA) I allele, was found to have harmful effects on the MS susceptibility (Zivkovic et al., 2014). In addition, genetic variation in the low-density lipoprotein-related protein 2 (LRP2) gene could lead to an increased relapse risk in MS (Zhou et al., 2017). A null genotype of glutathione S-transferase theta 1 (GSTT1), which is normally known to eliminate reactive oxygen species and toxins, was shown to frequently occur in patients with MS compared to controls showing that oxidative damage is one of an early event in MS (Zivkovic et al., 2014). Another environmental and genetic reason are variations in Vitamin D-receptor (VDR) gene which is associated with MS (Tajouri et al., 2005). Furthermore, smoking can drive the progression through the induction of nitric oxide (NO) (Hernan et al., 2001).

In addition to genetic and environmental influences, viruses have an impact on the onset and course of MS. Recent studies suggested an important role of viruses for the onset of MS (Marrie, 2004; Love, 2006), e.g. Epstein-Barr virus (Wagner et al., 2004), morbilli- and corona viruses (Sips et al., 2007), human herpesvirus 6 (Alvarez-Lafuente et al., 2004), herpes simplex-1 (HSV-1) (Christensen, 2005), varicella zoster virus (VZV) (Brudek et al., 2007) and human endogenous retrovirus type W (HERV-W) (Blond et al., 1999; Kremer et al., 2019). A hypothesis how viruses act during MS is the direct viral injury to the CNS which results in the exposition of myelin antigens and a subsequent autoimmune damage to myelin (Love, 2006). Furthermore, another hypothesis is that an immune response directed against the virus mediated via myeloid cells like brain-resident microglia could result in damage of myelin sheath (Love, 2006; Ginhoux and Prinz, 2015).

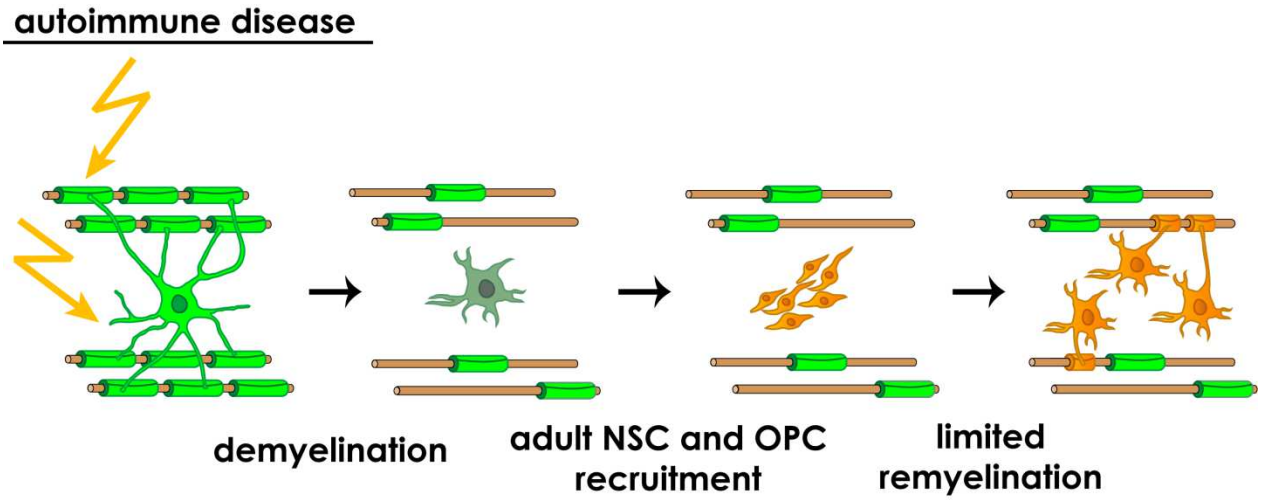

Figure 2.9: Demyelination and remyelination in an autoimmune disease affected nervous system

During an autoimmune disease such as multiple sclerosis (MS) myelin is lost due to invasion of activated immune cells which target myelinated axons and oligodendrocytes leading to cell death and demyelination (Kornek and Lassmann, 2003; Akkermann et al., 2016). However, the CNS has the potential for remyelination by recruitment of precursor cells (NSCs and OPCs) (Patani et al., 2007; Akkermann et al., 2016). Nevertheless, remyelination is limited due to multiple extrinsic and intrinsic inhibitory factors (Charles et al., 2002; Mi et al., 2005; Kremer et al., 2011).

Upon demyelination, OPCs become activated and recruited to the lesion sites with the potential to give rise to remyelinating oligodendrocytes (Figure 2.9) (Gensert and Goldman, 1997; Fancy et al., 2004). In addition, SVZ-derived NSCs are known to be a major contributor of remyelination (Figure 2.9) (Menn et al., 2006; Aguirre et al., 2007; Mecha et al., 2013; Xing et al., 2014; Brousse et al., 2015). For example,

during a Theiler's virus induced inflammation, which causes preclinical demyelination, an increased oligodendrocyte production and migration from the SVZ contributing to remyelination was observed in the corpus callosum (Mecha et al., 2013). Beside the contribution to remyelination, NSCs actively remyelinate most of the axons in the rostral part of the brain where the SVZ is located (Xing et al., 2014).

OPC activation is tightly regulated by different transcription factors, e.g. transcription factor 4 (TCF4) and Sox2 maintaining adult OPCs in the cell cycle in order to increase their numbers for repopulating demyelinated areas (Franklin and Ffrench-Constant, 2017). Activated astrocytes, microglia and macrophages guide OPCs directly to lesion sites by secretion of attractive factors (Franklin et al., 1997; Hammond et al., 2014; Moyon et al., 2015; Franklin and Ffrench-Constant, 2017). Probably, negative regulators of the differentiation such as the Notch- and the Wnt-pathway ensure that OPCs continue to proliferate during migration (Wang et al., 1998; Fancy et al., 2009; Franklin and Ffrench-Constant, 2017). Reaching the lesion site leads to OPCs´ cell cycle exit mediated by the proto-oncogene avian myelocytomatosis virus oncogene cellular homolog (myc), E2F1, interferon-gamma (IFN-gamma), cyclin-dependent kinase-2 (CDK2), cyclin-dependent kinase inhibitor 1B (p27Kip1), miR-297c-5p via interaction with cyclin T2 (CCNT2), SMAD3/4, forkhead box protein O1 (FoxO1) and specificity protein 1 (Sp1) (Casaccia-Bonnefil et al., 1997; Chew et al., 2005; Crockett et al., 2005; Tanner et al., 2011; Magri et al., 2014a; Magri et al., 2014b; Palazuelos et al., 2014; Kuypers et al., 2016) followed by differentiation initiated by an increased expression of Nkx2.2, Olig2, PDGF, Ascl1/Mash1, myelin transcription factor 1 (MyT1) and FGF (Fancy et al., 2004; Watanabe et al., 2004; Aguirre et al., 2007; Vana et al., 2007). In general, the process of remyelination is in many regards a recapitulation of the developmental occurring process of myelination (Franklin and Hinks, 1999; Fancy et al., 2011a). As during development, oligodendroglial cells migrate to their destinations of interest, stop proliferating and start myelination of axons (Baumann and Pham-Dinh, 2001), similar occurring during remyelinating phases (Chandran et al., 2008; Nait-Oumesmar et al., 2008) resulting also in an expression of CNPase, MBP, PLP and later on MOG (Lindner et al., 2008). Although many studies support the recapitulation hypothesis and some processes are similar, other studies refer to the differences between development and repair (Arnett et al., 2001; John et al., 2002; Michailov et al., 2004; Glezer et al., 2006; Kotter et al., 2006; Taveggia et al., 2008; Harrington et

al., 2010). Variations between these two processes exist regarding myelin thickness and diameter and role for inflammation (Arnett et al., 2001; Glezer et al., 2006; Harrington et al., 2010; Fancy et al., 2011b). Thus, newly formed myelin sheaths from OPCs are thinner and shorter compared to myelin sheaths formed during development (Blakemore, 1974; Ludwin and Maitland, 1984). However, myelin sheaths formed from aNSC-derived oligodendrocytes are thicker and longer compared to parenchymal-derived cells (Xing et al., 2014; Jadasz et al., 2018). While the process of remyelination itself appears to be effective, it is not the case in context of MS and disease progression (Prineas et al., 1993; Goldschmidt et al., 2009). Although OPCs are widely distributed, they do not differentiate efficiently due to inhibitory factors (Scolding et al., 1998; Wolswijk, 1998; Chang et al., 2000; Chang et al., 2002; Woodruff et al., 2004; Kuhlmann et al., 2008; Rivera et al., 2010; Kremer et al., 2011) such as the polysialic acid neural cell adhesion molecule (PSA-NCAM) which is expressed by demyelinated axons thereby preventing myelin-forming cells to attach to axons (Charles et al., 2002). The leucine rich repeat and immunoglobin-like domain-containing protein 1 (LINGO-1) is also known to negatively regulate OPC differentiation by activating RhoA and inhibiting Akt signaling pathways (Mi et al., 2005; Mi et al., 2013). Furthermore, it was demonstrated that TGF-β1 is upregulated in the brain of MS patients and that it restricts OPC maturation through Jagged1/Notch1 signaling (John et al., 2002). In addition, the G-protein coupled receptor 17 (GPR17), a factor that negatively regulates the oligodendroglial differentiation via Id2, was found to be upregulated during MS and in an EAE model (Chen et al., 2009). The nuclear factor-1A is expressed in MS lesions as well as in lysolecithin induced demyelination and is known to suppress OPC differentiation during remyelination presumably via the suppression of the myelin genes such as MBP, MAG and PLP (Fancy et al., 2012). Another negative regulator of OPC myelination is hyaluronan which is upregulated in MS lesions (Sloane et al., 2010). Blocking the chemokine receptor CXCR2, which is a negative regulator of OPC maturation results in an improved myelin repair after a cuprizone-induced demyelination and using an EAE mice model (Liu et al., 2010).

2.3.1.1 Stem cell-based therapies in demyelinated diseases

Stem cell-based therapies are considered as promising treatment option for multiple sclerosis which could counteract the reduced myelin repair capacity through the

replacement of damaged myelin-forming oligodendrocytes, modifying immune reactions and activation of endogenous repair mechanisms (Sarkar et al., 2017). Transplantation of different stem cell types such as NSCs, ESCs, OPCs, hematopoietic stem cells (HSCs) or MSCs were performed in experimental demyelination models such as the shiverer demyelination mutant mouse, EAE and cuprizone-mediated demyelination (Kim and de Vellis, 2009) to investigate the capacity to remyelinate or differentiate into oligodendrocytes (Soleimani et al., 2016). It has been demonstrated that transplantation of rat oligodendrocyte progenitor cells in myelin-deficient rat brains results in myelination of axons and enhanced oligodendroglial marker expression (Espinosa de los Monteros et al., 1997). Furthermore, oligodendrocytes or their progenitors transplanted into the CNS of neonatal or adult canine myelin mutants results in repair of large demyelinated brain areas (Archer et al., 1997). Furthermore, OPCs or oligodendrocytes isolated from rat or mice were transplanted into the brain of dysmyelinated mutants or chemical induced demyelination lesion showing an induced remyelination in the demyelinated regions (Franklin and Blakemore, 1997; Learish et al., 1999; Zhang et al., 1999; Espinosa de los Monteros et al., 2001; Ben-Hur et al., 2003; Kim and de Vellis, 2009). OPCs isolated from fetal human brains have been transplanted into a shiverer mouse brain resulting in remyelination (Gumpel et al., 1987; Seilhean et al., 1996; Windrem et al., 2004).

Beside oligodendrocytes or OPCs, NSCs were transplanted to promote remyelination. For example, NSC transplantation into the shiverer mouse brain resulted in widespread engraftment with remarkable MBP expression (Yandava et al., 1999). Olig2-overexpressing NSCs differentiated into actively remyelinating oligodendrocytes after transplantation into the corpus callosum after cuprizone-mediated demyelination (Copray et al., 2006). Furthermore, transplantation of human NSCs overexpressing Olig2 into shiverer mouse brains revealed an extensive myelination (Kim and de Vellis, 2009). In another study using shiverer mice, MS patient-derived iPSCs were used to produce oligodendrocytes which can then achieve myelination *in vivo* (Douvaras et al., 2014).

While these studies specifically focused on stem cell and cell replacement for successful myelination, remyelination and myelin repair, many studies focus on the modulation and influence on the immune response during a demyelination disease. Through hematopoietic stem cells (HSCs) which substantially alter the characteristics

of T cell responses and other immune reactivities, it is possible to modulate the immune system and therefore potentially improve the clinical course of autoimmunity, including aberrant immune responsivity in MS (Sarkar et al., 2017). Modifications in the immune regulatory compartment, such as a transient increase in regulatory forkhead box P3 (FoxP3)+ T cells was reported after using autologous hematopoietic stem cell transplantation (Radaelli et al., 2014). Furthermore, hematopoietic stem cell transplantation normalize the immunoregulatory gene expression (Arruda et al., 2015; de Paula et al., 2015).

Beside of NSC, OPC and HSC, MSCs were transplanted and used as treatment approach for MS disease models. MSCs are known to have immunomodulatory properties as well as the potential to activate endogenous repair mechanisms. MSCs are known to stimulate local proliferation of endogenous neural precursors, promote CNS neurite outgrowth and remodeling, secrete various trophic factors and protective antioxidants and are able to reduce gliotic scar formation (Kemp et al., 2010; Shen et al., 2011; Rice et al., 2013; Sarkar et al., 2017). MSCs transplanted into a demyelinated spinal cord resulted in remyelinated areas (Akiyama et al., 2002) as well as functional recovery after transplantation into an injured spinal cord (Chopp et al., 2000). It was shown that MSC administration before a disease onset obviously ameliorates EAE (Zappia et al., 2005). Similarly, the therapeutic scheme was effective when MSCs were administered at disease onset or at the peak of disease resulting in a decreased inflammatory infiltrates and demyelination in mice. Furthermore, the T cell response to MOG and mitogens was inhibited after MSC treatment (Zappia et al., 2005). Transplantation of bone marrow-derived MSCs into an EAE model suppressed the chronic EAE (Karussis et al., 2008). MSCs were shown actively migrate to white matter lesions and furthermore reduce lymphocytic infiltration with a significant preservation of axons (Karussis et al., 2008). Although an improvement of the EAE disease process takes place, only small numbers of transplanted cells were detectable, thus the amelioration appeared to be mainly due to a MSC induced peripheral or systemic immunomodulatory effect (Gordon et al., 2008). Such neuroprotective function and further immunomodulatory properties were also reported by Kassis and colleagues after intravenous and interventricular MSC injection (Kassis et al., 2008). Furthermore, MSCs seem to promote functional recovery of chronic and relapse-remitting models of mouse EAE. Interestingly, injection of MSC-secreted factors (MSC-CM) into an EAE model led to functional

repair by promoting the development of oligodendrocytes and neurons (Bai et al., 2012), thus MSC-secreted factors could also be used to promote regeneration and oligodendrogenesis (Uccelli et al., 2008; Uccelli et al., 2011) providing a promising therapeutic approach.

2.4. Aim of this thesis

In demyelinating diseases such as multiple sclerosis myelin repair activities were observed based on recruitment, activation and differentiation of resident progenitor and stem cells. However, the overall degree of successful remyelination remains limited.

In this regard, MSC-secreted factors have been shown to substantially enhance oligodendrogenesis of cultured primary aNSCs which could provide a novel pro-myelinating treatment approach promoting myelin repair. The underlying mechanisms and the nature of MSC-secreted factors fostering an oligodendroglial differentiation have, however, not been identified so far. Furthermore, the functional degree of MSC-CM depending lineage instruction *in vivo* remains to be established as well. In this regard the purpose of the present study was

1. To determine whether MSC-derived factors can stimulate aNSCs towards a pro-oligodendroglial lineage *in vivo* after transplantation into different rodent CNS regions.
2. To identify pro-oligodendroglial proteins by a comparative mass spectrometry analysis and subsequent functional evaluation of promising candidate proteins in culture and *in vivo.*
3. To test the effect of trophic factors emanating from MSCs derived from human iPSC as an attractive alternative source to replace bone marrow stem cells to prevent invasive preparation procedures.

3. Material and methods

3.1 Material

3.1.1 Equipment

Table 3.1: Laboratory equipment

Equipment	Company
7900HT Fast Real-Time PCR System	Thermo Fisher Scientific, Darmstadt, Germany
Acclaim PepMap100 trap column	Thermo Fisher Scientific, Darmstadt, Germany
Acclaim PepMapRSLC (2 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 25 cm length)	Thermo Fisher Scientific, Darmstadt, Germany
Autoclave GLA30	Fritz Gössner GmbH, Hamburg, Germany
Axioplan 2 Fluorescence microscope	Carl Zeiss, Oberkochen, Germany
BBD 6220 CO_2 incubator	Thermo Fisher Scientific, Darmstadt, Germany
Centrifuge	Heraeus Holding GmbH, Hanau, Germany
Cryostat CM3050S	Leica Biosystems Nussloch GmbH, Nussloch, Germany
Hamilton syringe (10 µl Neuros Model 1701 RN, ga 33, L 0-20 mm)	Hamilton Bonaduz AG, Bonaduz, Switzerland
Herasafe HSP 12 sterile bench	Heraeus, Hanau, Germany
Leo 906 E electron microscope	Carl Zeiss, Oberkochen, Germany
mass spectrometer (Orbitrap Fusion Lumos mass spectrometer)	Thermo Fisher Scientific, Darmstadt, Germany
micro centrifuge	Carl Roth GmbH & Co.KG, Karlsruhe

microtome (Microm HM 650 V)	Thermo Fisher Scientific, Darmstadt, Germany
Minishaker MS2; vortexer	IKA® Works, Inc. Wilmington, USA
NanoDrop ND 1000	PeqLab, Erlangen, Germany
Nikon eclipse TE200 microscope	Nikon, Tokyo, Japan
Nucleofector® II	Lonza, Basel, Switzerland
ProScan Slow Scan CCD camera	Carl Zeiss, Oberkochen, Germany
stereotaxic instrument (motorized)	Neurostar, Tubingen, Germany
Thermomix	Biotech international, Worcester, UK
Thoma counting chamber (Depth: 0.100mm; Area: 0.0025 mm^2)	Optik Labor, Görlitz, Germany
Ultimate 3000 RSCLnano System	Thermo Fisher Scientific, Darmstadt, Germany
Veriti Thermocycler	Applied Biosystems, Foster, City, USA
Water bath	GFL, Burgwedel, Germany
Zeiss LSM 510 microscope	Zeiss, Oberkochen, Germany

3.1.2 Chemicals

Table 3.2: Chemicals

Substance	Company
2-propanol	Merck, Darmstadt, Germany
4.6-diamidino-2-phenylindole	Roche Diagnostic GmbH, Mannheim, Germany
α-MEM	Gibco Cell Culture, Thermo Fisher Scientific, Darmstadt, Germany (for rat MSCs); Sigma-Aldrich Chemie GmbH, Steinheim, Germany (for human MSCs)
Accutase	PAA Laboratories, Pasching, Austria
Acetone	Merck, Darmstadt, Germany
Ampicillin	Roche, Mannheim, Germany
B27	Gibco BRL, Thermo Fisher Scientific, Darmstadt, Germany
Baytril	Bayer Health Care; Leverkusen, Germany
β-mercaptoethanol	Sigma-Aldrich Chemie GmbH, München, Germany
Bovine serum albumin	Thermo Fisher Scientific, Darmstadt, Germany
Cacodylate buffer	Carl Roth, Karlsruhe, Germany
Citiflour mounting medium	Citifluor, London, UK
Dithiothreitol	Serva Electrophoresis, Heidelberg, Germany
Diaminobenzidine–HCl	Vector Laboratories, Burlingame, USA
DMEM	Gibco Cell Culture, Thermo Fisher Scientific, Darmstadt, Germany

DMSO	Carl Roth, Karlsruhe, Germany
DNase I	Worthington Biochemicals
Dispase II	Boehringer, Ingelheim, German
EGF	R&D Systems, Wiesbaden-Nordenstadt, Germany
Ethanol ≥ 96%	Merck, Darmstadt, Germany
F12	Gibco Cell Culture, Thermo Fisher Scientific, Darmstadt, Germany
Fetal calf serum Lot No. 10500-064	Gibco Cell Culture, Thermo Fisher Scientific, Darmstadt, Germany
FGF-2	R&D Systems, Wiesbaden-Nordenstadt, Germany
Glutamax ®	Thermo Fisher Scientific, Darmstadt, Germany
Glycerol	Sigma-Aldrich, St. Louis, USA
Heparin	Sigma-Aldrich Chemie GmbH, München, Germany
Immu-mount	Thermo Fisher Scientific, Darmstadt, Germany
Insulin	Sigma-Aldrich Chemie GmbH, München, Germany
Iodacetamide	Sigma-Aldrich Chemie GmbH, München, Germany
Isoflurane	Piramal-Healthcare, Mumbai, India
Ketamine	Pfizer Pharma PFE GmbH, Berlin, Germany
Lauroyl sarcosinate	Sigma-Aldrich Chemie GmbH, München, Germany
Laminin	Sigma-Aldrich Chemie GmbH, München, Germany
L-Glutamine	Gibco BRL, Thermo Fisher Scientific,

	Darmstadt, Germany
LiChrosolv® water	Merck, Darmstadt, Germany
Matrigel	Becton Dickinson, Heidelberg, Germany
Minimum Essential Alpha Medium	Gibco BRL, Thermo Fisher Scientific, Darmstadt, Germany
Neurobasal medium	Gibco BRL, Thermo Fisher Scientific, Darmstadt, Germany
Normal goat serum	Sigma-Aldrich, St. Louis, USA
OCT Tissue Tek®	HistoService R. Jung, Nussloch, Germany
Papain	Worthington Biochemicals, Lakewood, USA
Paraformaldehyd	Merck, Darmstadt, Germany
Penicillin / Streptomycin	Gibco BRL, Thermo Fisher Scientific, Darmstadt, Germany (for rat MSCs); Thermo Fisher Scientific, Darmstadt, Germany (for human MSCs)
phosphate buffered saline	PAA Laboratories, Pasching, Austria
Poly-L-ornithine hydrochloride	Sigma-Aldrich Chemie GmbH, München, Germany
Putrescine	Sigma-Aldrich Chemie GmbH, München, Germany
Progesterone	Sigma-Aldrich Chemie GmbH, München, Germany
RedDot 2	Biotium, Fremont, CA, USA
RNase-free water	Qiagen, Hilden, Germany
Rothi®-Liquid Barrier marker	Carl Roth, Karlsruhe, Germany
Rimadyl	Pfizer Deutschland GmbH, Berlin, Germany

Sodium selenite	Sigma-Aldrich Chemie GmbH, München, Germany
Sucrose	Carl Roth, Karlsruhe, Germany
StemMACS iPS-Brew XF, human	Miltenyi Biotec, Bergisch Gladbach, Germany
Streptavidin-biotin-peroxidase	Vector Laboratories, Burlingame, USA
Thiourea	Sigma-Aldrich Chemie GmbH, München, Germany
Transferrin	Sigma-Aldrich Chemie GmbH, München, Germany
transforming growth factor-β	Miltenyi Biotec, Bergisch Gladbach, Germany
Triton X-100	Sigma-Aldrich Chemie GmbH, München, Germany
Tris(hydroxymethyl)aminomethane	Carl Roth, Karlsruhe, Germany
Tris base	Sigma-Aldrich Chemie GmbH, München, Germany
Trypan blue	Sigma-Aldrich Chemie GmbH, München, Germany
Trypsin	Capricorn Scientific GmbH, Ebsdorfergrund, Germany
TrypLE Express	Thermo Fisher Scientific, Waltham, MA
Urea	Sigma-Aldrich Chemie GmbH, München, Germany
Xylazine	Sigma-Aldrich Chemie GmbH, München, Germany

3.1.3 Consumables

Table 3.3: Consumables

Material	Company
Coverslips 13 mm Ø	Glaswarenfabrik Karl Hecht GmbH & Co. KG, Sondheim, Germany
Cryotubes 2 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Culture dishes mm, 24 wells 100 mm Ø petri dish 5 mm Ø petri dish	Greiner Bio-One GmbH, Frickenhausen, Germany
Culture flaks 25 cm^2 75 cm^2	Sarstedt AG und Co. KG, Nümbrecht, Germany
Filter 0.2 µm filtropur S	Sarstedt AG & Co. KG, Nümbrecht, Germany
Micro-reaction tubes 0.5 ml 1.5 ml 2.0 ml	Sarstedt AG und Co. KG, Nümbrecht, Germany
Pipette tips / Stripettes 10 µl 10/20 µl 100 µl 200 µl 1000 µl 5 ml 10 ml 25 ml	• Eppendorf GmbH, Wesseling-Berdorf, Germany • Gilson, Limburg-Offheim, Germany • Greiner Bio-One GmbH, Frickenhausen, Germany
Pasteur pipettes	Brand GmbH & CO., Wertheim, Germany
Glass slides	• Engelbrecht Medizin und

	Labortechnik, Edermünde, Germany • Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany • Thermo Fisher Scientific, Darmstadt, Germany
Tubes 5 ml 15 ml 50 ml	Sarstedt AG und Co. KG, Nümbrecht, Germany

3.1.4 Kits

Table 3.4: Kits

Kit	Company
Amaxa® Rat NSC Nucleofector® Kit	Lonza, Basel, Switzerland
Endo Free Plasmid Maxi Kit	Qiagen, Hilden, Germany
Pierce™ 660 nm Protein Assay	Thermo Fisher Scientific, Darmstadt, Germany

3.1.5 Primary Antibodies

Table 3.5: Primary Antibodies

Antibody	RRID	species / isotype	dilution	Company
Caspr (clone K65/35)	AB_10671175	mouse IgG	1:500	UC Davis/NIH NeuroMab Facility, California, USA
Chordin	AB_2079795	mouse IgG	25 µg/ml	R&D Systems, Wiesbaden-Nordenstadt, Germany
CNPase	AB_2565362	mouse IgG	1:500 ICC	Biolegend, San

				Diego, USA
GFAP	AB_10013382	rabbit IgG	1:1000 ICC 1:10000 IHC	DAKO Agilent, Santa Clara, USA;
GFP	AB_10000240	chicken IgG	1:2000	Aves Labs, Tigard, US
GFP	AB 91337	rabbit IgG	1:100 EM	Merck Millipore, Burlington, USA
GSTπ	AB_10615079	rabbit IgG	1:500 ICC 1:4500 IHC	ENZO Life Sciences GmbH, Lörrach, Germany
Isotype control IgG antibody	AB_354267		25 µg/ml	R&D Systems, Wiesbaden-Nordenstadt, Germany
MBP	AB_2565364	mouse IgG	1:200 ICC	Biolegend, San Diego, USA
MBP	AB_92396	rabbit IgG	1:500 ICC 1:250 IHC	Merck Millipore, Burlington, USA
MBP	AB_325004	rat IgG	1:250 ICC 1:250 IHC	Bio-Rad Laboratories, Inc., Hercules, California, USA;
Neurofilament	AB_306298	rabbit IgG	1:2500	Abcam, Cambridge, UK
NG2	AB_11213678	rabbit IgG	1:100	Merck Millipore, Burlington, USA
Olig2	AB_570666	rabbit IgG	1:1000-1:2000 IHC	Merck Millipore, Burlington, USA
TIMP-1	AB_355455	Rat IgG	25 µg/ml	R&D Systems, Wiesbaden-Nordenstadt, Germany

3.1.6 Secondary Antibodies

Table 3.6: Secondary Antibodies

Antibody	isotype	Conjugate	Dilution	Company
α-chicken	goat IgG	Alexa 488	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-mouse	goat IgG	Alexa 488	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-mouse	goat IgG	Alexa 594	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-mouse	goat IgG	Alexa 647	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-rabbit	goat IgG	Alexa 488	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-rabbit	goat IgG	Alexa 594	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-rabbit	goat IgG	Alexa 647	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-rabbit	goat IgG	biotinylated	1:50 EM	Vector Laboratories, California, USA
α-rat	goat IgG	Alexa 488	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)
α-rat	goat IgG	Alexa 594	1:500 ICC 1:200 IHC	Life technologies GmbH (Carlsbad, USA)

3.1.7 DNA expression vectors / plasmids

Table 3.7: DNA expression vectors / plasmids

Name	Company
pcDNA3.1-HygB-citrine	see (Heinen et al., 2008)
UbC-StarTrack plaminds pCMV-hyPBase	see (Figueres-Oñate et al., 2016)

UbC-EGFP	

3.1.8 Software

Table 3.8: Software and software-components

Software	Company
Adobe Illustrator CS2	Adobe Systems, San Jose, USA
Adobe Photoshop CS2	Adobe Systems, San Jose, USA
Axiovision 4.2 software	Zeiss, Oberkochen, Germany
Endnote X7.7.1 & X9	Thomas Reuters, New York City, USA
GraphPad PRISM software 5.01	GraphPad Software, Inc, La Jolla, USA
iTEM (Soft Imaging System)	Carl Zeiss, Oberkochen, Germany
ImageJ software	National Institute of Health, Rockville, USA
MaxQuant (version 1.6.6.0)	MPI for Biochemistry, Planegg, Germany
MS Office 2007 /2016	Microsoft Corporation, Redmond, USA
OutCyte 1.0 Server	Molecular Proteomics Laboratory (MPL), Biologisch-Medizinisches Forschungszentrum (BMFZ), Heinrich-Heine-University, Düsseldorf, Germany
Primer Express 3.0.1	Applied Biosystems, Foster City, USA
StereoDrivesoftware	Neurostar, Tubingen, Germany
Zen black software	Zeiss, Oberkochen, Germany
Zen blue software	Zeiss, Oberkochen, Germany

3.2 Composition of cell culture media, buffers and other reagents

3.2.1 Cell culture

Table 3. 9: cell culture media and reagents

Media	Contents	Concentration
α-MEM of rat MSCs	MEM Alpha Medium FCS Pen Strep	 10% (v/v) 1% (v/v)
α-MEM of human MSCs	MEM Alpha Medium FCS Pen Strep Glutamax	 10% (v/v) 1% (v/v) 1% (v/v)
DMEM	Dulbecco's Modified Eagle's Medium	
F12	Ham's Nutrient Mixture F12	
NB not all	Neurobasal medium Pen Strep L-Glutamine B27	 1% (v/v) 1% (v/v) 1%? (v/v)
NB all	NB not all EGF FGF Heparin	 20 ng/ml 20 ng/ml 2 µg/ml
N2-medium	DMEM F12 Insulin Sodium selenite Putrescine Progesterone transferrin	75% 25% 5 mg/ml 30 nM 100 µM 20 nM 5 µg/ml
PBS	1x Phosphate buffered saline	PAA Laboratories, Pasching, Austria

PDD	papain dispase II DNase I $MgSO_4$ HBSS	0.01% 0.1% 0.01% 12.4 mM
StemMACS iPS-Brew XF, human	Pen Strep	1% (v/v)

3.2.2 Immunocytochemistry

Table 3.10: Buffer/reagents for immunocytochemistry

Buffer / reagent	Content	Concentration
Antibody solution	PBS NGS Triton-X 100	 1% (v/v) 0.03% (v/v)
Blocking solution	PBS NGS Triton-X 100	 1% (v/v) 0.1% (v/v)
Paraformaldehyde	PBS PFA	 4%
PBS		

3.2.3 Immunohistochemistry

Table 3.11: Buffers/reagents for immunohistochemistry

Buffer / reagent	Content	Concentration
Antibody solution	TBS BSA	 10% (v/v)
Blocking solution	TBS BSA Triton-X 100	 10% (v/v) 4% (v/v)

Blocking solution2	PBS NGS Triton-X 100	 10% (v/v) 0.2% (v/v)
Tris-buffered saline	TRIS NaCl H_2O	12.2% (w/v) 18% (w/v)
TBS-T	TBS Triton-X 100	1% 0.2% (v/v)
Paraformaldehyde	PBS PFA	 4%

3.3 Animals and preparation

3.3.1 Animals used for preparation of adult neural stem cells and mesenchymal stem cells

Primary aNSCs and MSCs were isolated from wildtype female Wistar rats (8-10-weeks old, ~210g body weight) bred in the ZETT – Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben Düsseldorf.

3.3.2 Transplantation animals

Experiments were performed with wildtype female C57BL/6 mice (*Mus musculus*; C57Bl/6, 13-14 weeks old, ~18-21g body weight) and rats (Wistar, 10-12 weeks old, between 210 and 230g) provided by Janvier Labs (Saint-Berthevin, France) accommodated in the ZETT. All transplantation experiments were approved by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; Az.: 84-02.04.2015.A239; Az.: 84-02.04.2015.A525) and carried out in accordance with ethical care. All rodents were housed in a pathogen-free facility with 12 h light/dark cycle and supplied with food/water *ad libitum.*

Table 3.12: Animal numbers used for stereotactic transplantation into the mouse brain

transplantation into the mouse brain						
pre-stimulation time-points	days post transplantation					
	4		7		14	
	α-MEM	MSC-CM	α-MEM	MSC-CM	α-MEM	MSC-CM
	animal number				animal number	
1	4	4	3	3	4	4
3	5	4	4	4	6	6

Table 3.13: Animal numbers used for stereotactic transplantation into the rat brain

transplantation into the mouse brain		
pre-stimulation time-points	days post transplantation	
	14	
	α-MEM	MSC-CM
	animal number	
3	1	1

Table 3.14: Animal numbers used for stereotactic transplantation into the rat spinal cord

transplantation into the rat spinal cord						
pre-stimulation time-points	days post transplantation					
	14		28		14	
	α-MEM	MSC-CM	α-MEM	MSC-CM	IgG crtl MSC-CM	anti-TIMP1 MSC-CM
	animal number				animal number	
3	4	5	6	4	2	4

3.4 Cell culture

3.4.1 Adult rat neural stem cell preparation and neurosphere culture

The 8-10 weeks old rats were anesthetized and sacrificed by means of cervical dislocation. For the preparation of the adult neural stem cells, SVZ was dissected and

collected in PBS. The collected tissue (SVZ) was centrifuged at 800rpm (~ 140g) at 4°C for 5 min, mechanically unstitched via a scalpel and washed in ice-cold PBS. For enzymatic digestion, 10 ml PDD was added and incubated for 30 min at 37°C with 10 min trituration steps in between. After digestion, cells were centrifuged at 140g for 5min and 4°C, washed two times with NB not all and centrifuged again. The cells were resuspended in NB all and seeded in T25 culture flaks at 37°C in a humidified incubator with 5% CO_2. Every two to three days the medium was changed until the cells formed neurospheres. Cells were passaged using accutase for 10-30 min at 37°C and seeded in T75 flaks. Accutase was stopped with an equal volume of NB all medium. The dispersal step was repeated once a week and aNSCs were plated with a density of 0.5-2 x 10^6 cells/flask.

3.4.2 Differentiation assay of adult neural stem cells and transfection

For differentiation assay, aNSCs were dispersed with accutase 10-30 min with minimum three trituration steps and plated on a poly-L-ornithine/laminin (100 µg/ml and 12 µg/ml, respectively) coated 24 well culture dish with acid pre-treated 13 mm glass cover slips. For transplantation experiments, cells were seeded on poly-L-ornithine/laminin coated 100 mm Ø petri dishes. Poly-L-ornithine (diluted in ddH_2O) coating was performed over night at 37°C. Cover slips were washed three times with H_2O and coated with laminin (in PBS) over night at 4°C. aNSCs were incubated for 24 hours in proliferation medium NB all containing EGF (20 ng/ml), FGF-2 (20 ng/ml) and heparin (2 µg/ml). For differentiation initiation, medium was changed to control Minimum Essential alpha Medium (α-MEM), rat mesenchymal stem cell conditioned medium (MSC-CM), to human fetal H1555 MSC-CM (hMSC-CM) and/or to induced MSC-CM (iMSC-CM; from iH1555, see chapter 4.9). All media contained 10% FBS. aNSCs were incubated with differentiation medium for different time points (1 or 3 and 7 days for cell culture experiments; 1 and 3d for transplantation). For cell visualization of transplanted cells, aNSC were co-transfected with the UbC-StarTrack plasmids; pCMV-hyPBase and UbC-EGFP (Figueres-Oñate et al., 2016) using the rat neural stem cell nucleofectorTM kit (Lonza, Basel, Switzerland). Transfected cells were seeded with a concentration of 3x10^6 cells in one T75 culture flasks in 10 ml NBall medium and cultured for seven days at 37°C in a humidified incubator with 5% CO_2. Two or four days before transplantation, aNSCs were seeded on poly-L-ornithine/laminin (100 µg/ml and 12 µg/ml, respectively) coated petri-dishes (35 x

10mm) for 24h in NBall medium before changing to control α-MEM/10% FBS or MSC-CM.

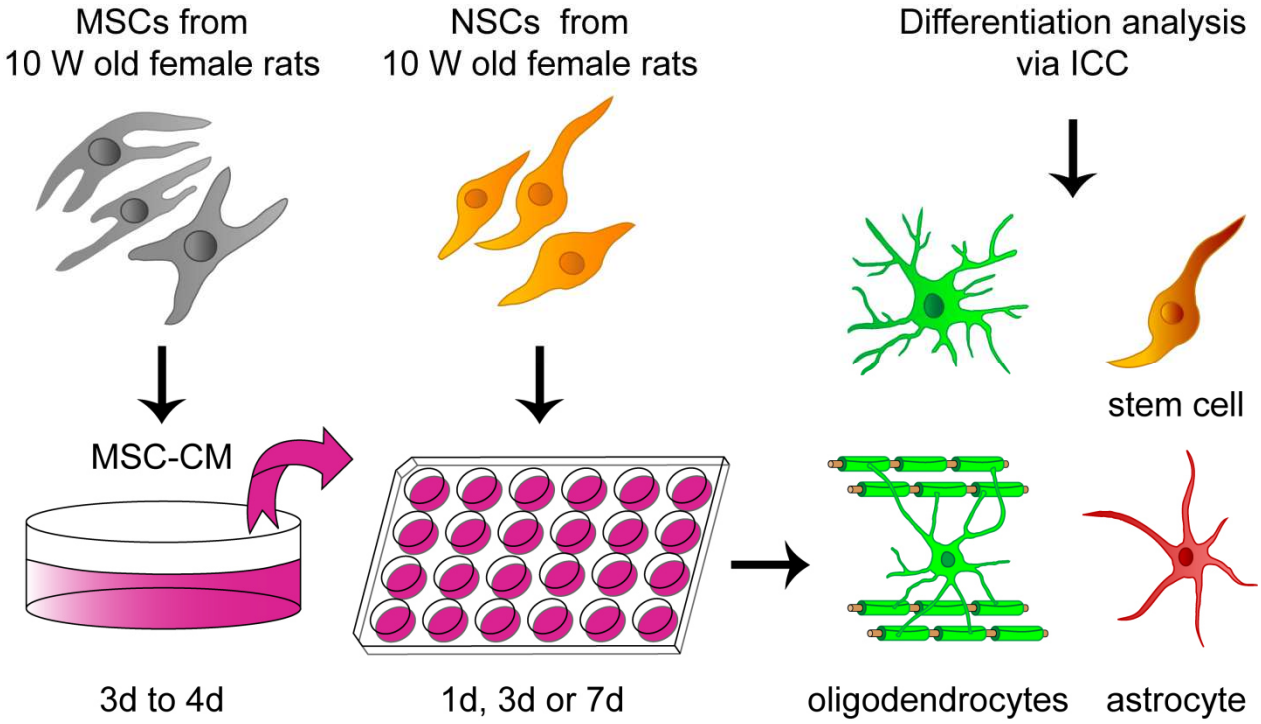

Figure 3.1: Cell culture workflow for differentiation

Bone marrow derived MSCs and aNSCs were generated both from ~10-week old female Wistar rats. MSCs were pre-incubated for 3-4 day in α-MEM before using the MSC-conditioned medium (MSC-CM) for the stimulation of aNSCs. For differentiation, aNSCs were plated on poly-L-ornithine/laminin 24h before stimulation with MSC-CM or control α-MEM. aNSCs were then stimulated for 1, 3 or 7 days in culture. Same procedure was performed for all investigated media (α-MEM, MSC-CM, hMSC-CM and iMSC-CM). Subsequently, immunocytochemistry was performed to characterize the aNSC differentiation.

3.4.3 Adult mesenchymal stem cell preparation and culture

Female Wistar rats (age of 8-10 weeks) were anesthetized and sacrificed by means of cervical dislocation. Femur and tibia were dissected and the surrounding tissue was removed. The tibial plateau and femoral head were cut off and the bone marrow was scoured out with α-MEM containing 10% FBS using a cannula. After repeated flushing to dissociate bone marrow clumps, the bone marrow histoid were centrifuged at 1700rpm (~800g) at 4°C for 10 min and incubated at 37°C with 5% CO_2 in a humidified incubator for 3-5 days. The medium was changed and non-adherent cells were recovered by centrifugation (800g, 10min, 4°C). The pellet was resuspended in 10 ml α-MEM-10%FBS and incubated for 3-5 days. Adherent cells were incubated in 10 ml fresh α-MEM-10%FBS for 3-5 day until a confluent layer (90-100%) of cells

was reached. Cells were trypsinized using 0.25% trypsin-EDTA solution (3min at 37°C) and stopped with an equal volume of α-MEM. The detached cells were recovered by centrifugation, resuspended in 10ml α-MEM, counted and seeded in α-MEM-10%FBS with a density of $4x10^5$ cell/100mm Ø culture dish. After three to four days incubation the MSC-conditioned medium (MSC-CM) was removed and cells were cultured in α-MEM/10%FBS for another three to four days. The corresponding supernatant was filtered (0.2 µm filtropur S filter) and used as rat mesenchymal stem cell conditioned medium (MSC-CM) for aNSC stimulation. MSCs were used from passage 3 to 10.

3.4.4 Human fetal mesenchymal stem cells

Human MSC line H1555 was derived from male fetal femoral bones (provided by Richard Orrefo, Southampton University, UK). The conditioned medium was provided by the Institute for Stem Cell Research and Regenerative Medicine (ISRM, Prof. James Adjaye, Heinrich-Heine-Universität). Briefly, human fetal (h) femur-derived MSCs (H1555) were obtained following informed written patient consent at 55 days post conception. Approval was obtained by the Southampton and South West Hampshire Local Research Ethics Committee (LREC 296100). The experimental use of hMSCs was approved by the ethics commission of the Heinrich-Heine-University, Düsseldorf. Their derivation and characterization has been described previously (Mirmalek-Sani et al., 2006). Human fetal MSCs were cultured in α-MEM which was supplemented with 10% FBS (Gibco), 1% GlutaMAX® and 1% penicillin/streptomycin Medium was sterile filtered and replenished every three days and kept as conditioned medium (hMSC-CM) for further experiments.

3.4.5 Human iPSC-derived mesenchymal stem cell

The conditioned medium derived from iPSC-derived MSC was provided by the Institute for Stem Cell Research and Regenerative Medicine (ISRM, Prof. James Adjaye, Heinrich-Heine-Universität).

The non-viral generation of iPSCs from human fetal MSCs (H1555) was performed as described before (Megges et al., 2015) as well as the generation of iPSCs derived iMSCs (Spitzhorn et al., 2018). iPSCs were cultured without feeder cells on Matrigel using StemMACS iPS-Brew XF, human, with 1% penicillin/streptomycin in humidified

atmosphere with 5% CO2 at 37°C, with daily medium change. Confluent cell cultures were changed to α-MEM (see above) and the pluripotent cells were treated daily with 10 mM of the transforming growth factor-β receptor inhibitor SB-431542 for 14 days. The emerged iMSCs were trypsinized using TrypLE Express for 10 min at 37°C, centrifuged at 300 g for 5 min, and seeded onto uncoated culture dishes in MSC expansion medium (α-MEM), which led to the depletion of cells with maintained iPSC characteristics. Further passaging was carried out as described above when cells reached about 95% confluency. Cells were seeded at $1x10^6$ cells per T175 culture flask. Medium was sterile filtered and replenished every three days and kept as conditioned medium (iMSC-CM) for further experiments.

3.4.6 Antibody blocking

For blocking experiments, aNSCs were seeded on acid-pretreated and poly-L-ornithine/laminin coated glass cover slips ($5x10^4$ cells/coverslip) and cultured in NBall medium for 24h. To neutralize TIMP-1 or chordin (Chrd), MSC-CM was incubated with an rat anti-TIMP-1 antibody (25 µg/ml) or a mouse anti-chordin (25 µg/ml) for 1h at 37°C prior application to aNSCs. Isotype control IgG antibody (25 µg/ml) was used as control. In addition, both antibodies were also applied to normal control medium (α-MEM/10% FCS) in order to exclude non-specific antibody effects. After 3 days of incubation with α-MEM/10% FCS or MSC-CM, aNSCs were fixed using 4% paraformaldehyde (PFA) and subjected to immunocytochemical staining.

3.5 Stereotactic cell transplantations

3.5.1 Stereotactic cell transplantations into the rodent brain

For transplantation experiments, rat aNSCs were detached with accutase and kept at room temperature in their respective media. For transplantation into the mouse and rat brain, aNSCs were centrifuged for 5 min at 140g, washed once in PBS and finally resuspended in PBS to a concentration of 1×10^5 cells/µl. Stereotactic cell transplantations into the Wistar rat brains were performed by our cooperation partners Veronica Estrada and Christine Bütermann from the Department of Neurology, Medical Faculty, Prof. Hans Werner Müller, Heinrich-Heine-University, Düsseldorf, Germany. Recipient C57Bl/6J mice or Wistar rats were deeply

anesthetized using isoflurane or sevoflurane inhalation. The skull was exposed (Figure 3.2) using a scalpel and holes were drilled at 0.7 mm (anterior-posterior), ± 1 mm (medial-lateral) for mice and at 0.7 mm (anterior-posterior), ± 1.5 mm (medial-lateral) for rats. Approximately 0.75 µl of the cell suspension was injected in the white (corpus callosum) and grey (cortex) matter of the mice brains (13-14 weeks old) according to Viagno et al., 2013 (Vigano et al., 2013) and approximately 1 µl of the cell suspension was injected into the white (corpus callosum) and grey (cortex) matter of the rat brains (10-12 weeks old). Transplantations were performed with a Hamilton syringe (10 µl Neuros Model 1701 RN, ga 33, L 0-20 mm) at 0.7 mm (anterior-posterior), ± 1 mm (medial-lateral), 2.1 to 1.8 mm (dorsal-ventral) for mice and at 0.7 mm (anterior-posterior), ± 1.5 mm (medial-lateral), 3 to 1.4 mm (dorsal-ventral) for rats relative to Bregma using a motorized robot stereotaxic instrument and Stereo Drive software for mice or a manual stereotaxic instrument for rats. Postoperative care comprised an analgesic treatment (Rimadyl, 5mg/kg) for three days starting on the day of operation for mice. Postoperative care for rats included prophylactic oral antibiotic treatment (Baytril, 0.4 ml/kg) for seven days and analgesic treatment (Rimadyl, 5 mg/kg) for three days starting on the day of surgery. For tissue collection, mice were deeply anesthetized with isoflurane and transcardially perfused with 20 ml cold PBS followed by 20 ml 4% PFA. Rats were deeply anesthetized using a mixed solution containing ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and were transcardially perfused with 200 mL 4°C ice-cold PBS followed by 400 mL 4% PFA. Mouse brains were post-fixed overnight in 4% PFA at 4°C, followed by 24 to 48 h cryoprotective dehydration in 30% sucrose (in PBS) at 4°C. Rat brains were harvested and post-fixed overnight in 4% PFA at 4°C, followed by 24 h washing in PBS and 24 to 48 h cryoprotection in 30% sucrose (in PBS) at 4°C. Rat as well as mouse brains were then embedded in Tissue-Tek OCT, frozen, and stored at -30°C until preparation of 10 µm (mouse brain) and 14 µm (rat brain) sections using a cryostat. Sections were stored at -30°C.

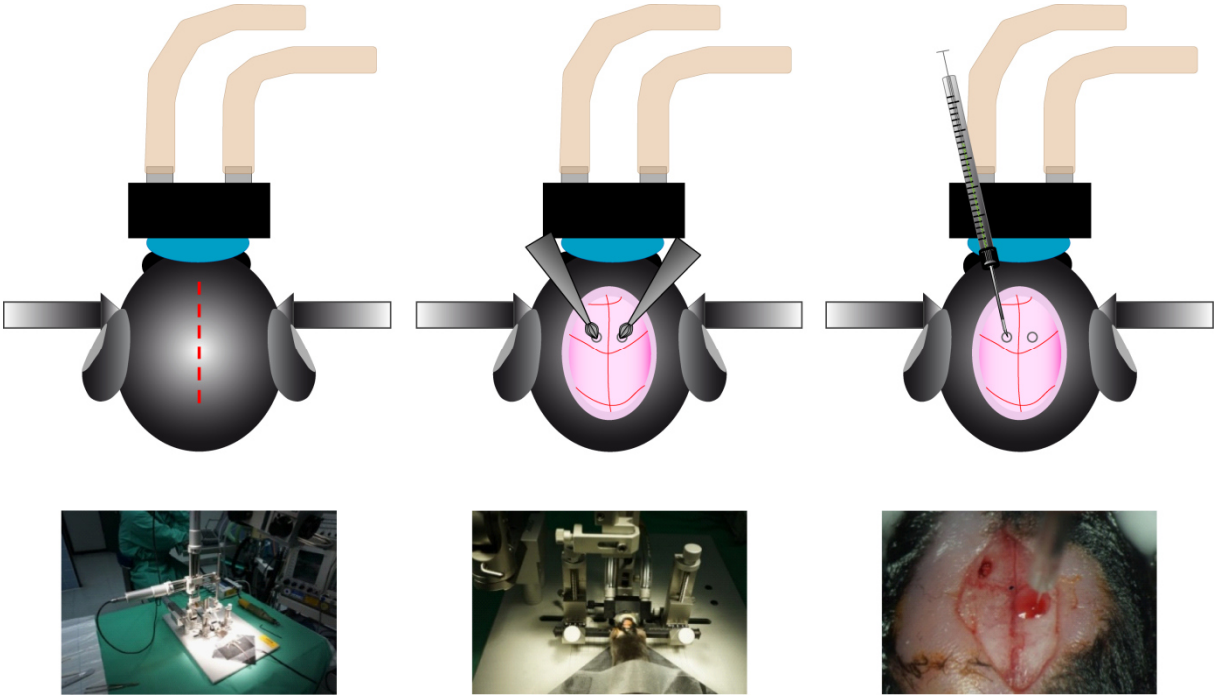

Figure 3.2: Schematic presentation of surgery/transplantation procedure of stem cell transplantations into the mouse brain

13-14 weeks old mice were deeply anesthetized using isoflurane or sevoflurane inhalation and part of the head fur was removed. The skull was exposed using a scalpel and holes were drilled at 0.7 mm (anterior-posterior), ± 1 mm (medial-lateral). Afterwards, ~0.75 µl of the cell suspension was injected using a Hamilton in 7 steps at 2.1 to 1.8 mm (dorsal-ventral) relative to Bregma with a motorized robot stereotaxic instrument to reach the white (corpus callosum) and grey (cortex) matter of the mice brains.

3.5.2 Stereotactic cell transplantations into the spinal cord

Stereotactic cell transplantations into the Wistar rat spinal cords were performed by our cooperation partners Veronica Estrada and Christine Bütermann from the Department of Neurology, Medical Faculty, Prof. Hans Werner Müller, Heinrich-Heine-University, Düsseldorf, Germany. For spinal cord transplantations, adult female Wistar rats were used. Rat aNSC, either stimulated with control α-MEM, rat MSC-CM, anti-TIMP-1 blocked MSC-CM or IgG control blocked MSC-CM, were used and transplantation was performed at thoracic level eight (Th8) using a Hamilton syringe (10 µl Neuros Model 1701 RN, ga 33, L 0-20 mm) attached to a small animal stereotaxic instrument (David Kopf Instruments, Tujunga, USA). Injections were performed at 0.1 mm lateral to the midline and 1.1 mm to 0.8 mm dorsal-ventral from the dural surface. At each transplantation site 1 µl containing either 1x 10^5 control α-MEM pre-stimulated (3 days) or MSC-CM pre-stimulated (3 days) aNSCs in PBS were injected slowly within 4 min. For TIMP-1 experiments 1 µl containing either 1x 10^5 IgG control blocked MSC-CM pre-stimulated (3 days) or anti-TIMP-1 blocked

MSC-CM pre-stimulated aNSCs in PBS were injected slowly within 4 min per each transplantation site. Postoperative care included prophylactic oral antibiotic treatment (Baytril, 0.4 ml/kg) for seven days and analgesic treatment (Rimadyl, 5 mg/kg) for three days starting on the day of surgery. After 14 or 28 days post-operation, animals were deeply anesthetized using a mixed solution containing ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and transcardially perfused with 200 ml 4°C ice-cold PBS followed by 400 ml 4% PFA. Spinal cords were harvested and post-fixed overnight in 4% PFA at 4°C, followed by 24 h washing in PBS and 24 to 48 h cryoprotection in 30% sucrose (in PBS) at 4°C. Spinal cords were then embedded in Tissue-Tek OCT, frozen, and stored at -30°C until preparation of 14 µm sections using a cryostat. Sections were stored at -30°C.

3.6 Immunofluorescence staining

3.6.1 Immunocytochemistry

For immunocytochemistry, cells were fixed with 4% PFA for 10 min and washed three times with PBS. After blocking of non-specific binding sites and permeabilization for 45 min with 1% normal goat serum (NGS) and 0.1% Triton-X100 in PBS, primary antibodies (see Table 6) were incubated in 1% NGS (in PBS, 0.03% Triton-X100) at 4°C overnight. Primary antibody incubation was followed by three washing steps with PBS and incubation with secondary antibodies (see Table 3.5) conjugated with either Alexa Fluor594 or Alexa Fluor488 in 1% NGS (in PBS, 0.03% Triton-X100) supplemented with 4´,6-diamidin-2-phenylindol (0.02 µl/ml DAPI) at room temperature for 90 min. Cells were mounted with Citifluor and images were taken using a Zeiss Axioplan2 microscope.

3.6.2 Immunohistochemistry

For immunohistochemistry, frozen brain slices were dried at RT for 10-20 min and washed in ddH_2O for 5 min and dehydrated for 5 min with ice cold acetone. After dehydration slices were washed in TBS and TBS-T for 5 min each. Brain slices were outlined with Rothi®-Liquid Barrier marker and blocked with blocking solution containing 10% BSA and TBS-T for 30 min at RT. Primary antibodies (see Table 6) were diluted in antibody solution containing 10% BSA and TBS and incubated

overnight at 4°C or RT. Afterwards, slices were washes twice in TBS for 5 min. Secondary antibodies (see Table 3.6) and the nuclear dye DAPI (1:50) or RedDot 2 (1:200) were diluted in TBS and incubated on the slices for 30 min at RT. After incubation slices were finally washed in TBS-T and TBS for 5 min each and mounted with immu-mount.

3.6.3 Documentation und image editing

Documentation of the staining was performed by using the Axioplan 2 fluorescence microscope (Zeiss) for ICC with the Axiovision 4.2 software (Zeiss) and the Zeiss LSM 510 microscope (Zeiss) for IHC using the Zen black / blue software (Zeiss). For immunocytochemistry, 10 areas per coverslip using a 40x lens were recorded. The editing of the photos was performed with the help of the Zeiss LSM Image Browser and Illustrator CS2 (Adobe). For quantitative analyses, Image J Software (National Institute of Health (NIH) USA) (Schindelin et al., 2012; Schneider et al., 2012) was used to count positive cells which were normalized to the total cell number (DAPI-positive cells).

For immunohistochemistry, a minimum of 100 GFP+ cells per condition were taped with a magnification of 200-400-times. Immunohistochemical staining on brain and spinal cord sections was performed using the corresponding centers of transplantation. An average of 25 brain sections and 13 spinal cord sections per marker and per time-point were analyzed including 3 brain slices or 2 spinal cord slices per slide. Fluorescently labeled cells were counted on each section and the mean number per slide (containing 3 brain or 2 spinal cord sections, respectively) was calculated leading to an average value for each animal.

The editing of the photos was performed with the help of the Zeiss Zen black and blue software, Photoshop CS2 and Illustrator CS2 (Adobe). For quantitative analyses, Image J Software and the Zen blue software (Zeiss) was used to count positive cells which were normalized to the total number of GFP-positive cells.

3.6.4 Statistical evaluation of immunofluorescence stainings

For statistical analysis, two-way analysis of variance (ANOVA) with Bonferroni posttest was applied using GraphPad Prism 5.0c software. Statistical significance thresholds were set as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. All data are shown

as mean values ± standard error of the mean (SEM) and “n” represents the number of independent experiments performed.

3.7 Immunoelectron microscopy

For immunoelectron microscopy of transplanted aNSCs in the spinal cords, adult Wistar rats were perfused with 4% PFA in 1 M cacodylate buffer, the spinal cords were harvested and post-fixed overnight in 4% PFA in 1M cacodylate buffer. Following post fixation, spinal cords were washed over night in PBS, embedded in 6% agarose in cacodylate buffer and coronal sections (50 µm) were cut in PBS using a microtome (Microm HM 650 V, Thermo Fisher Scientific).

Staining and immunoelectron microscopy was performed by our cooperation partners Dr. Janos Groh and Prof. Martini (Department of Neurology, University Hospital Würzburg, Würzburg). Free-floating sections were stained with rabbit anti-GFP antibody (see Table 6) in 1% BSA in PBS overnight at 4°C and afterwards visualized using a biotinylated secondary antibody (see Table 3.6) and streptavidin-biotin-peroxidase (Vector Laboratories) complex using diaminobenzidine–HCl (Vector Laboratories) and H_2O_2. Sections were osmicated and processed for light and electron microscopy by dehydration and embedding in Spurr's medium. Ultrathin sections (50 nm) were mounted to copper grids, counterstained with lead citrate, and investigated using a ProScan Slow Scan CCD camera connected to a Leo 906 E electron microscope (Zeiss, Jena, Germany) and corresponding software iTEM (Soft Imaging System). Transplanted cells were identified based on GFP-immunoreactivity (indicated by dark precipitates).

3.8 Preparation and analysis of mesenchymal stem cell secretome and proteome

Secretome, proteome analysis and data validation was performed in cooperation Prof. Kai Stühler of the Molecular Proteomics Laboratory (MPL, Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-University, Düsseldorf).

To identify enriched proteins using N2 serum free conditioned MSCs, the abundances of proteins identified in the MSC proteome were compared to the secreted proteins in the MSC-CM (relative protein abundances).

For LC-MS/MS analysis, mesenchymal stem cells were prepared as described before (see 3.4.3). Confluent MSCs were washed three times with PBS to remove the FBS and then incubated for another 48h in serum free N2-medium (Schira et al., 2015; Schira-Heinen et al., 2019; Schira et al., 2019) consisting of DMEM and Ham's Nutrient Mixture F12 containing 5 mg/ml insulin, 30 nM sodium selenite, 100 µM putrescine, 20 nM progesterone and 5 µg/ml transferrin. Medium was changed again after 48h to further exclude FBS effects and then incubated for another 48h in N2-medium.For mass spectrometry analysis, four independent biological replicates of MSCs were used. To analyze secreted proteins a trichloroacetic acid (TCA) precipitation was performed. Therefore, conditioned medium was centrifuged with 950g at 4° for 10 min and filtered through a 0.2 µm filter (Pall Acrodisc). The medium was mixed with 10% sodium lauroyl sarcosinate (Sigma-Aldrich Chemie GmbH) up to a final concentration of 0.1%. Ice-cold TCA (Sigma-Aldrich Chemie GmbH) was added (7.5% final concentration), incubated for 1 h on ice and centrifuged at 7100g at 4°C for 10 min. After centrifugation the supernatant was removed, the pellet was resuspended in 1 ml ice-cold acetone and centrifuged again. Again, the supernatant was removed and the pellet was dried at room temperature. The pellet was dissolved in lysis buffer consisting of 30 mM Tris base (Sigma-Aldrich Chemie GmbH), 7M urea (Sigma-Aldrich Chemie GmbH,), and 2M thiourea (Sigma-Aldrich Chemie GmbH). The protein content was determined by the Pierce™ 660 nm Protein Assay (Thermo Fisher Scientific).

To analyze the corresponding cell proteome, MSCs were washed three times with cold PBS after removing the conditioned medium for secretome analysis. Afterwards, 2 ml cold PBS was added and cells were detached using a cell scraper. Cell suspension was transferred to a tube and centrifuged with 800g at 4°C for 5 min. The supernatant was removed and cell pellets were immediately frozen and stored at -80°C. For cell lysis, MSCs incubated in lysis buffer (described above), the lysate was sonicated 6 × 10 sec, centrifuged at 16000g for 15 min and the protein concentration of the supernatant was determined.

For in-gel digestion, 5 µg total protein of each sample (secretomes as well as proteomes) was used for short SDS-gel-electrophoresis (5 mm running distance, 10 min) and stained with Coomassie brilliant blue. The resulting lanes were excised, washed, reduced with 10 mM DTT (Serva Electrophoresis, Heidelberg, Germany) and alkylated with 55 mM iodacetamide (Sigma-Aldrich Chemie GmbH). Proteins

were digested with 0.1 µg trypsin (Serva Electrophoresis) over night at 37°C and peptides were extracted, dried and finally resuspended in 0.1% trifluoroacetic acid (TFA).

3.8.1 Liquid chromatography and mass spectrometric analysis

For separation of the extracted peptides a Ultimate 3000 RSCLnano System (Thermo Fisher Scientific) with an Acclaim PepMap100 trap column (3 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 2 cm length, Thermo Fisher Scientific) as a precolumn using 0.1% TFA as a mobile phase and an Acclaim PepMapRSLC (2 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 25 cm length, Thermo Fisher Scientific) analytical column was used. For peptide separation a constant 300 nl/min flow rate for analytical separation and a 2 h gradient of 0.1% formic acid (Fluka) to 0.1% formic acid/60% acetonitrile was used. Peptides were eluted via nano electrospray ionization into the mass spectrometer (Orbitrap Fusion Lumos mass spectrometer, Thermo Fisher Scientific) operated in positive mode and advanced peak determination enabled. Precursor mass spectra were recorded in the orbitrap analyzer within a mass range of 200–2,000 m/z and a resolution of 120,000 (maximum ion time 60 ms, automatic gain control target value 250000, profile mode). For a maximum of two seconds, precursors with charge states +2 to +7 and a minimum intensity of 5000 were isolated within a 1.6 m/z isolation window and fragmented via higher-energy collisional dissociation. MS/MS spectra were recorded in the linear ion trap in centroid mode with a maximal ion time of 50 ms and a target value for the automatic gain control set to 10,000. The scan rate was “rapid” and already fragmented precursors were excluded from further isolation for the next 60 s.

3.8.2 Analysis of mass spectrometric data

To identify peptides and proteins, the MaxQuant environment was used (version 1.6.6.0, MPI for Biochemistry, Planegg, Germany). If not stated otherwise, the identification was done with standard parameters. Searches were performed using the rat UP000002494 proteome data set (29951 entries) downloaded on 10th April 2019 from the UniProt Knowledgebase using tryptic specificity (cleavage behind R and K) with a maximum of two missed cleavages sites. Carbamidomethylation at cysteines was considered as fixed and methionine oxidation was set as variable modification. A first search was performed with 20 ppm precursor mass tolerance. Peptides identified with high confidence were then used for recalibration using the

“software lock mass” feature of MaxQuant (Cox et al., 2011). Thereafter, a main search was conducted with a precursor mass tolerance of 4.5 ppm. The mass tolerance or fragment spectrum was set to 0.5 Da. Peptides and proteins were accepted at a false discovery rate of 1%. The “match between runs” function was enabled as well as label-free quantification.

Corresponding secretomes and proteomes were compared to identify enriched proteins in the secretome probably representing secreted proteins according to Grube and colleagues (Grube et al., 2018). After MaxQuant based database search and quantification, quantitative protein level data were analyzed within the Perseus framework (version 1.6.6.0, MPI for Biochemistry, Planegg, Germany). Proteins identified only by site or marked as contaminant (from the MaxQuant contaminant list) as well as reverse hits were excluded. Proteins with at least two identified peptides and a minimum of three valid values in at least one group were considered. Calculations were done on normalized intensities (LFQ intensities) as provided by MaxQuant, and missing data were imputed before statistical analysis by values from a downshifted normal distribution (width 0.3 standard deviations, down-shift 1.8 standard deviations). The significance analysis of microarrays (SAM) method (Tusher et al., 2001) was applied on $\log_2$-transformed values using a S0 constant of 0.8 and a 5% false discovery rate based cutoff. Presented fold changes have been calculated as difference from mean values of $\log_2$ transformed intensities.

To predict unconventionally secreted proteins, the OutCyte prediction tool was used (Zhao et al., 2019), classically secreted proteins were selected based on the annotation from UniProtKB (signal peptide predicted, but neither a transmembrane domain nor a kdel sequence present). Gene Ontology (GO) biological process (GOBP) and cellular compartment (GOCC) were used for categorical annotations of proteins identified in the MSC-derived secretome and annotation enrichments were calculated by Fisher’s exact tests. Enrichment associated p-values were adjusted via the method of Benjamini and Hochberg.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD018231.

4. Results

4.1 Stereotactic cell transplantations of MSC-CM pre-stimulated aNSCs into the mouse brain

Mesenchymal stem cells are known to have strong regenerative, anti-inflammatory and tumor-promoting properties (Uccelli et al., 2008; Maffioli et al., 2017; Spitzhorn et al., 2017; Teixeira et al., 2017; Baberg et al., 2019; Jungbluth et al., 2019; Timaner et al., 2020). Furthermore, MSC-secreted factors favor maturation and oligodendroglia differentiation of primary cultured OPCs and aNSCs (Rivera et al., 2006; Jadasz et al., 2013; Jadasz et al., 2018). To investigate the potential of MSC-secreted factors to promote aNSCs to generate oligodendroglial cells *in vivo*, cell transplantations into different CNS tissues were performed. MSC-CM pre-stimulated GFP-expressing aNSCs were transplanted into young adult mouse brain to test their differentiation and tissue integration capacity in two different brain environments (grey and white matter).

4.1.1 GFP-PiggyBac transfection did not alter the aNSCs differentiation potential after MSC-CM stimulation in culture

To exclude an influence of transfection on the aNSC differentiation and to evaluate the transfection efficiency as well as signal stability, transfections with the GFP-PiggyBac plasmid were performed. Transfected cells were either treated with control α-MEM or rat MSC-CM for 1, 3 and 7 days (Figure 4.1A,B). Quantitative analysis revealed no difference in GFP-expression between α-MEM and MSC-CM stimulation for all investigated time points (Samper Agrelo et al., 2020). A significant increase of up to 15% GFP-positive cells was detectable over time for both treatments compared to the 1-day time point (Figure 4.1B).

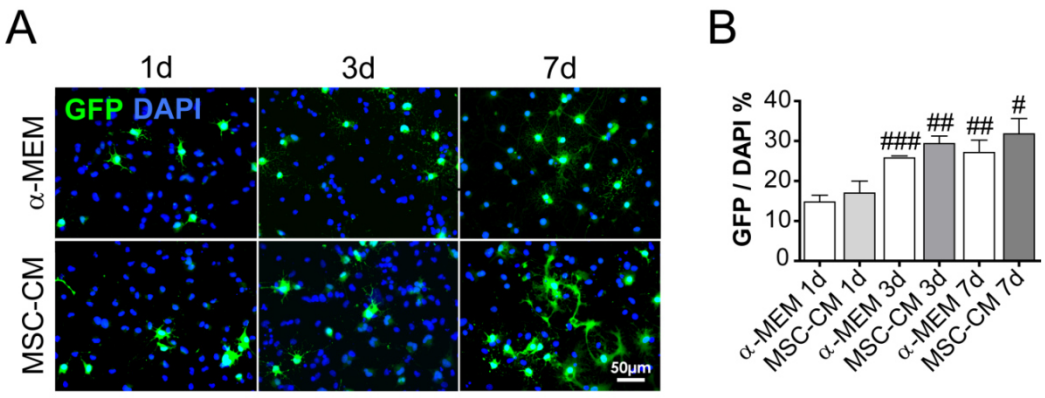

Figure 4.1: Transfection efficiency in aNSCs

(**A**) Representative pictures of transfected, GFP-positive cells (green) 1, 3 and 7 days after α-MEM or MSC-CM treatment in culture. (**B'**) Quantitative evaluation of the total number of GFP-positive cells in relation to the total number of cells (DAPI; blue) showing no difference in the cell number between α-MEM or MSC-CM but showing an increase of GFP-positive cells over time. For statistical analysis a two-way ANOVA with Bonferroni posttest was used: # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$, 1 to 3 or 1 to 7 days (α-MEM or MSC-CM). Data are shown as mean values ± SEM derived from n=3 experiments. Adapted from Figure S1: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

To analyze the influence of the PiggyBac plasmid transfection on cellular differentiation behavior, immunocytochemical staining for several differentiation markers was performed after a 1 to 7-day incubation either with α-MEM or MSC-CM. In comparison to non-transfected aNSCs (Jadasz et al., 2018), the differentiation capacity of aNSCs with GFP-PiggyBac plasmids was similar resulting in a stable nestin expression and a significant increase of oligodendroglial markers after 1 and 3-day MSC-CM stimulation compared to α-MEM controls (Figure 4.2) (Samper Agrelo et al., 2020). However, the nestin expression was diminished over the time for both control and MSC-CM (Figure 4.2A,A'). In addition, a significant increase of NG2-positive cells after 1 and 3 days of MSC-CM stimulation compared to the control was detected whereas the number of NG2-expressing cells was significantly decreased after 3 days compared to 1 day for both α-MEM and MSC-CM (Figure 4.2C,C').

While less NG2-positive cells were detected after 3 days, cells expressing CNPase and MBP were significantly increased after 3 days of MSC-CM treatment compared to 1 day (Figure 4.2D-E'). As seen in our previous study (Jadasz et al., 2018), MSC-CM stimulation resulted in a significant increase of GFAP-positive cells after 1 day and a slight decrease after 3 days. Comparison of 1 and 3 days of stimulation

revealed that α-MEM treatment results in an increase of GFAP-positive cells while MSC-CM stimulation led to a significant decrease after 3 days (Figure 4.2B,B'). These results reflect the differentiation pattern of non-transfected aNSCs (Jadasz et al., 2018) thus validating the MSC-CM dependent effects.

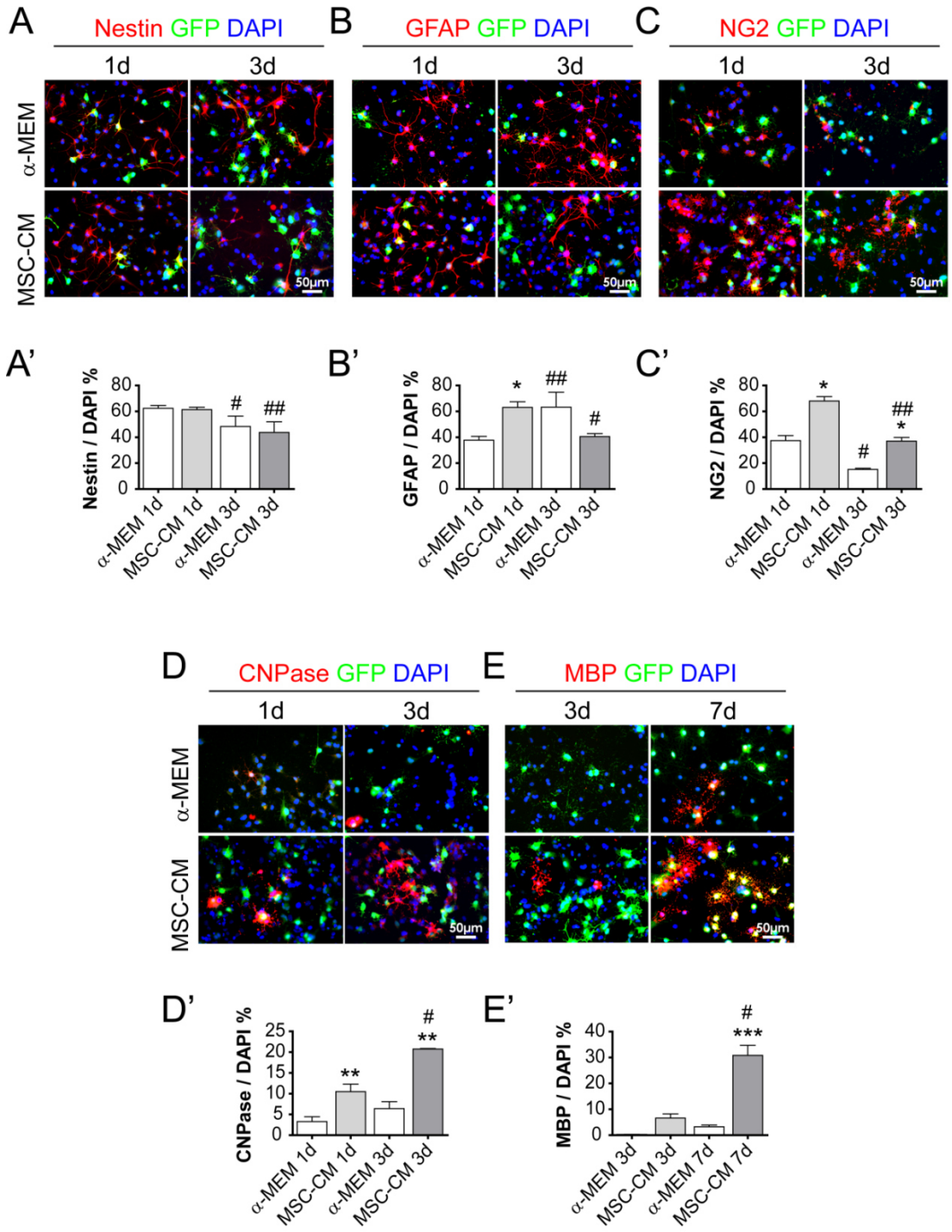

Figure 4.2: Differentiation pattern of Piggybac-transfected GFP-positive aNSCs after MSC-CM stimulation

(**A-E**) Representative pictures of GFP-positive cells (green) stained for stem cell, oligodendroglial and astroglial markers (red, respectively) at 1, 3 up to 7 days after MSC-CM

or control α-MEM treatment in culture (DAPI; blue). (**A'-E'**) Quantitative analysis of stem cell and glial cell markers revealed an increase of oligodendroglial markers (**C'-E'**) after MSC-CM stimulation and a decrease of GFAP-positive cells (**B'**) over time. For statistical analysis a two-way ANOVA with Bonferroni posttest was used: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, MSC-CM compared to the respective α-MEM control (1 or 3 or 7 day pre-stimulation); # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$, 1 to 3 or 1 to 7 days (α-MEM or MSC-CM). Data are shown as mean values ± SEM derived from n=3 experiments. Adapted from Figure S1: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.1.2 Survival of MSC-CM stimulated aNSCs after transplantation into the adult mouse brain

To test which period of pre-stimulation with MSC-CM is needed to foster aNSCs into an oligodendroglial lineage differentiation *in vivo,* short and long-term pre-stimulation (1 and 3 days, respectively) with MSC-CM was performed before transplantation. Pre-stimulated NSCs were transplanted into grey (cortex) and white (corpus callosum) matter of 13-14 weeks old C57Bl/6 mice. First, the influence of pre-stimulation either with α-MEM or MSC-CM and regional dependency on the survival of transplanted aNSCs was investigated revealing that pre-stimulated aNSCs are able to survive up to 7 days post transplantation (pt) independent of the brain region (Figure 4.3). As shown by Samper Agrelo et al. (2020) immunohistochemical staining using an anti-GFP antibody revealed more GFP-positive cells were found after 4 days compared to 7 days pt independent of pre-stimulation duration and treatment. A significant decrease of GFP-positive cells was found after 7 days compared to 4 days pt with a 3-day pre-treatment with α-MEM (Figure 4.3B). In addition, a 3-day pre-stimulation (ps) seems to have a slight survival benefit compared to the 1-day pre-treatment (Figure 4.3A). Comparing the survival rate of NSCs transplanted into grey and white matter revealed no significant difference in the number of GFP-positive cells after 4 and 7 days post transplantation independent from pre-stimulation time point (Figure 4.3C,D). However, comparison of the pre-stimulation periods revealed that cells pre-treated with α-MEM survived significantly better in the grey matter (Figure 4.3C). This proportion was still visible for α-MEM after 7 days post transplantation (Figure 4.3D).

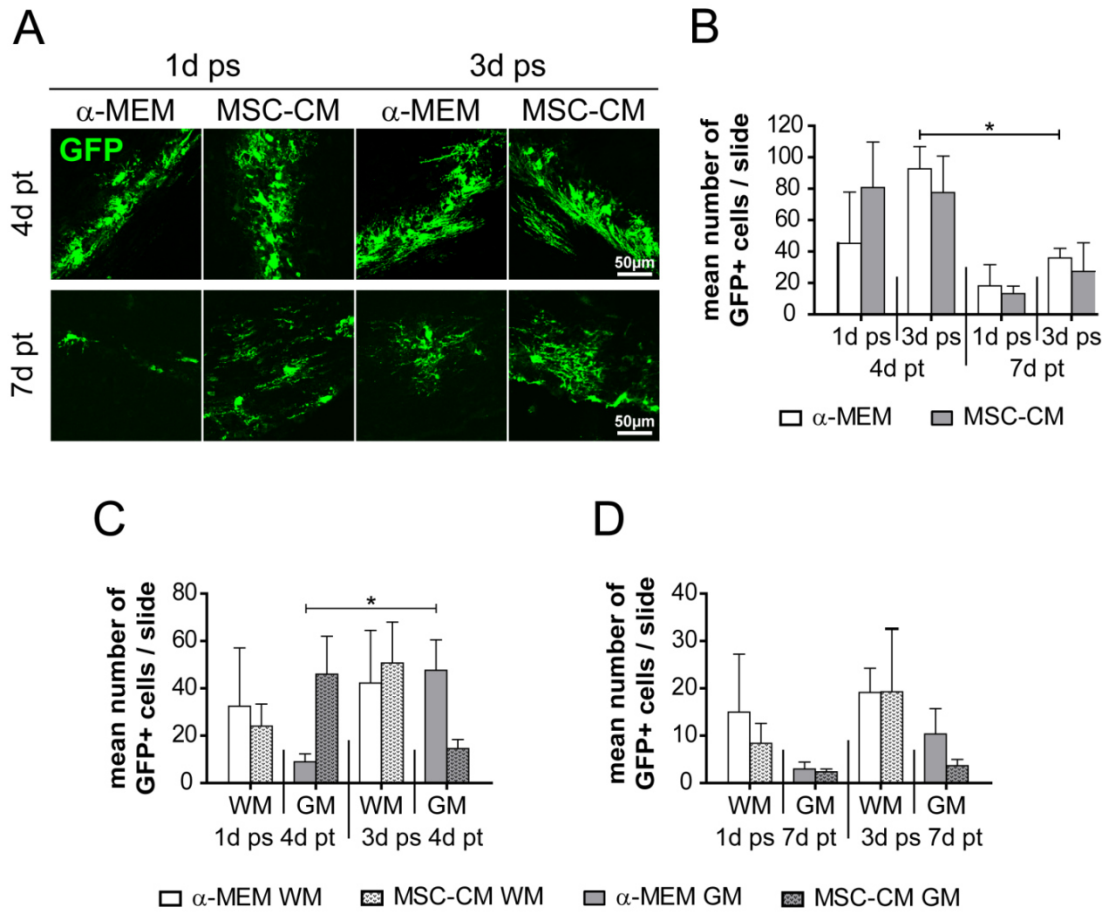

Figure 4.3: Survival rates of GFP-expressing pre-stimulated aNSCs after transplantation into the mouse brain

(**A**) Immunohistochemical staining of GFP-positive cells (green) 4 and 7 days after transplantation (pt) into the intact mouse brain (corpus callosum and cortex). aNSCs were pre-stimulated (ps) for 1 or 3 days with α-MEM or MSC-CM before transplantation. (**B**) Quantitative evaluation of the total number of GFP-positive cells per slide including both grey and white matter showed no difference in the cell number between α-MEM or MSC-CM or 1 and 3 day pre-stimulation. Comparison of 4 and 7 days pt revealed a decreased number of GFP-positive cells after a 3 day pre-treatment with α-MEM. (**C-D**) Comparing the number of GFP-positive cells per slide in the grey and white matter, no significant difference were found after 4 (**C**) and 7 days pt (**D**) following both pre-stimulation periods and conditions. Quantification of 1 and 3 day pre-stimulation revealed that more cells pre-stimulated with α-MEM survived in the grey matter after a 3-day pre-treatment. For statistical analysis, a two-way ANOVA with Bonferroni posttest was used: * $p \leq 0.05$, compared 4d pt to 7d pt in (**B**) and 1d ps to 3d ps in (**C**). Data are shown as mean values ± SEM derived from n=3-5 animals, for more details see Table 3.12. Adapted from Figure 1: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.1.3 Differentiation of MSC-CM stimulated aNSCs after transplantation into the mouse brain

To evaluate whether a pre-stimulation with MSC-secreted factors promotes oligodendroglial differentiation *in vivo*, the differentiation of GFP-positive aNSCs pre-stimulated for 1 or 3 days at 4 and 7 days after transplantation into the mouse brain was investigated by immunohistochemistry. Quantitative analysis revealed a significant increase of NG2-positive cells after 4 days with 1 and 3-day MSC-CM pre-stimulation (Figure 4.4A,A'). Approximately 30% of MSC-CM 1 or 3 days pre-stimulated aNSCs showed an Olig2 marker expression compared to α-MEM treated cells at 4 days pt (Figure 4.4B,B'). The expression of the mature oligodendrocyte marker glutathione-S-transferase-π (GSTπ) significantly raised to 79% after one day pre-stimulation and 74% after three days pre-stimulation compared to control treated cells (49% and 37%, respectively) at 4 days pt (Figure 4.4C,C'). These results were accompanied by a 40% increase of MBP-positive cells (Figure 4.4D,D') and a decrease of GFAP-positive cells after 1 and 3-day MSC-CM pre-stimulation (GFAP: 1d ps → 4d pt: α-MEM 57%, MSC-CM 31%; 3d ps → 4d pt: α-MEM 45%, MSC-CM 21%; Figure 4.3E,E'). No significant differences were detected between the investigated pre-stimulation time points for both α-MEM and MSC-CM pre-treated aNSCs (Samper Agrelo et al., 2020).

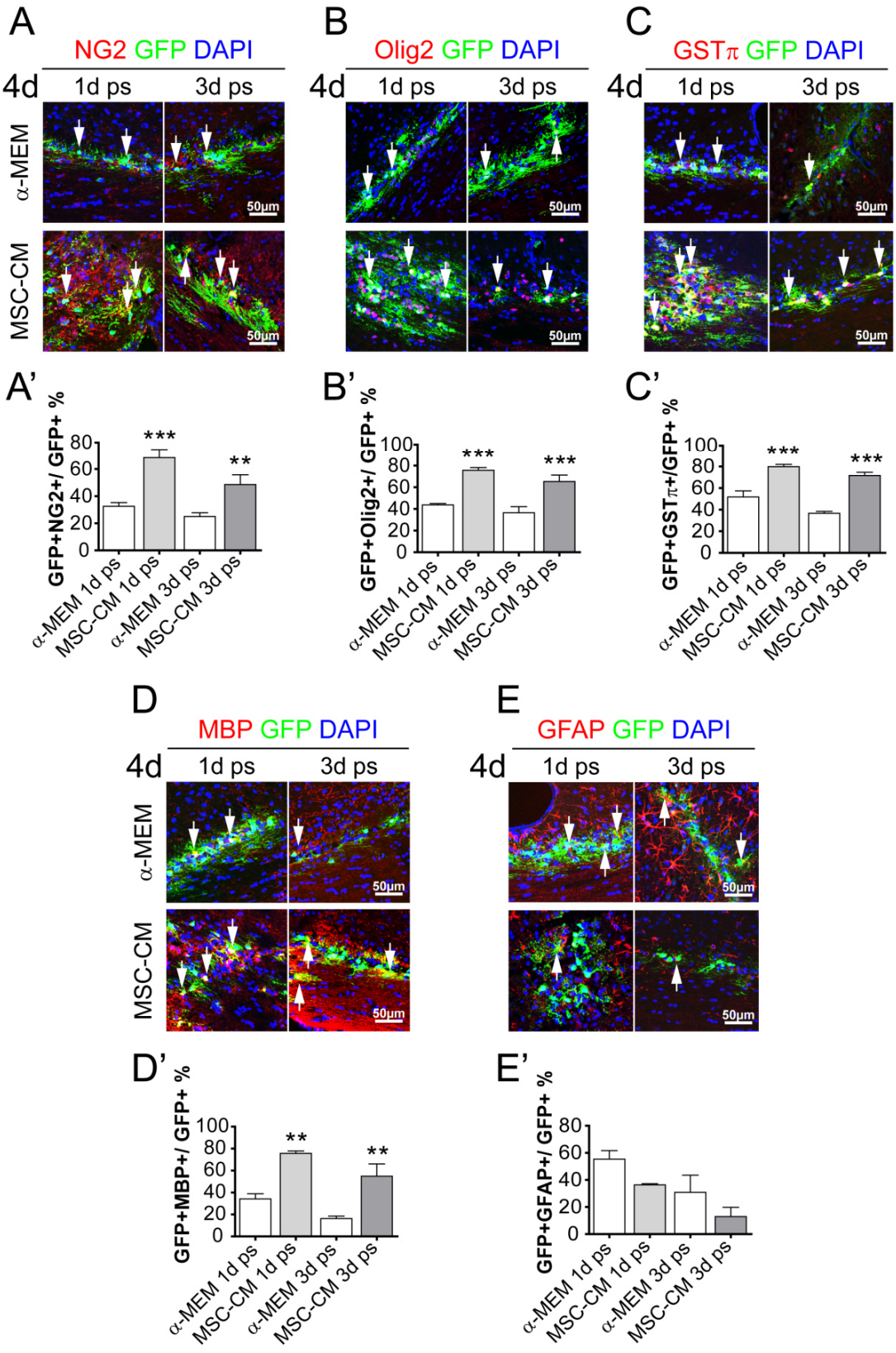

Figure 4.4: MSC-CM pre-stimulated aNSCs differentiate into maturing oligodendroglia early after transplantation into the mouse brain

(**A-E**) Representative images of α-MEM and MSC-CM 1 and 3-day pre-treated GFP-positive cells (green) expressing NG2 (**A**), Olig2 (**B**), GSTπ (**C**), MBP (**D**) or GFAP (**E**) 4 days post transplantation into the mouse brain. (**A'-E'**) Quantification of transplanted aNSCs expressing oligodendroglial markers revealed a significantly increased number of cells expressing NG2

(**A'**), Olig2 (**B'**), GSTπ (**C'**) and MBP (**D'**) after 4 days while the number of GFAP-positive cells were decreased (**E'**). For statistical analysis, a two-way ANOVA with Bonferroni posttest was used: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, MSC-CM compared to the respective α-MEM control (1 or 3 day pre-stimulation). Arrows point to GFP-positive cells (green) expressing the respective markers (red). Blue nuclei represent DAPI staining. Data are shown as mean values ± SEM derived from n=3-5 animals. Adapted from Figure 2: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

Similar effects were also observed at 7 days post transplantation (Figure 4.5). Here, 64% of the GFP-positive cells were NG2-positive after 1 day pre-stimulation with MSC-CM and 71% after 3 days pre-stimulation compared to controls (29% and 30%, respectively) (Figure 4.5A,A'). The same effect was observed for Olig2. MSC-CM pre-stimulation for 1 day led to an increase of 20% and pre-stimulation for 3 days to 35% more Olig2-positive cells compared to α-MEM treated cells, respectively (Figure 4.5B,B'). In addition, the number of mature oligodendrocytes was significantly increased at 7 day post transplantation. Quantification revealed that 74% of the GFP-positive cells expressed GSTπ (Figure 4.5C,C') and 88% were positive for MBP (Figure 4.5D,D') after pre-stimulation with MSC-CM for 1 day compared to α-MEM (GSTπ: 48%; MBP: 36%). Furthermore, 79% of GSTπ/GFP-positive and 78% MBP/GFP-positive cells were found after a 3-day MSC-CM pre-treatment compared to controls (GSTπ: 45%; MBP: 38%; Figure 4.5C-D'). This increase in oligodendroglial cells after one day as well as three days pre-stimulation with MSC-CM was accompanied with a significant decrease of 31% and 29% GFAP-positive cells compared to α-MEM, respectively (Figure 4.5E,E').

The here presented therefore data indicate that a 1-day long pre-stimulation with MSC-derived factors is sufficient to foster the oligodendroglial differentiation process of aNSCs after transplantation *in vivo*.

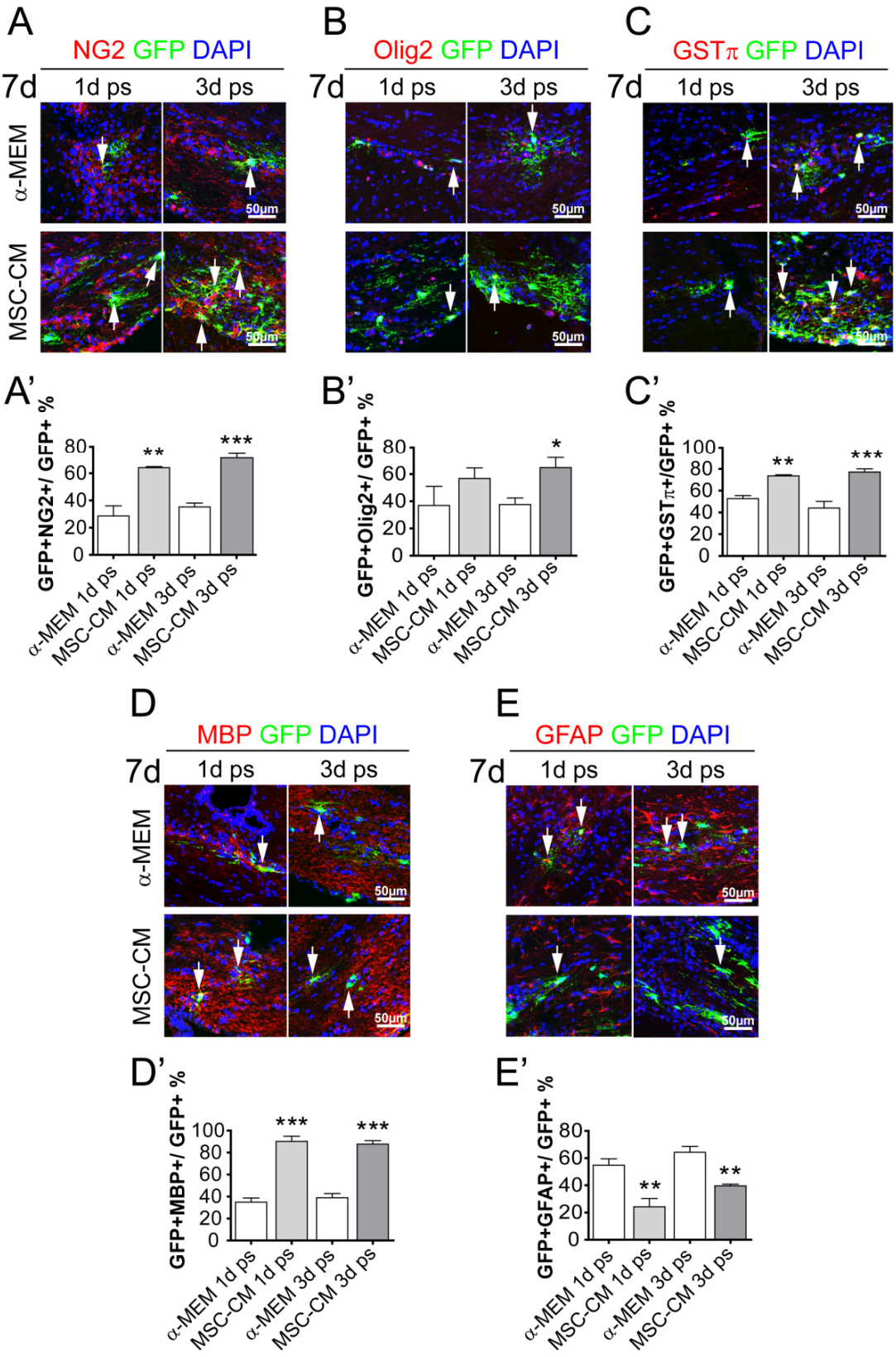

Figure 4.5: Mature oligodendrocytes differentiated from MSC-CM pre-stimulated aNSCs

(**A-E**) Representative images of 1 or 3-day pre-stimulated (α-MEM or MSC-CM) GFP-expressing cells positive for NG2 (**A**), Olig2 (**B**), GSTπ (**C**), MBP (**D**) and GFAP (**E**) 7 days post transplantation with the corresponding quantifications shown for NG2 (**A'**), Olig2 (**B'**), GSTπ (**C'**), MBP (**D'**) and GFAP (**E'**). Statistical analysis was performed by using a two-way

ANOVA with Bonferroni posttest: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, MSC-CM compared to the respective α-MEM control (1 or 3 day pre-stimulation). Arrows point to GFP-positive cells (green) expressing the respective markers (red). Blue nuclei represent DAPI staining. Data are shown as mean values ± SEM derived from n=3-4 animals. Adapted from Figure 2: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.1.3.1 Differentiation of MSC-CM stimulated aNSCs after transplantation into grey and white matter of the mouse brain

It was demonstrated that grey and white matter of the brain can differently affect OPC differentiation (Vigano et al., 2013) and that white matter localization promotes the differentiation of transplanted p57kip2 depleted aNSC towards myelinating oligodendrocytes at the expense of astrocytic glia cells (Beyer et al., 2020). To investigate these region dependent properties the differentiation capacity of MSC-CM pre-treated cells in the grey and white matter of the brain was compared. Comparison of control versus MSC-CM pre-stimulated NSCs revealed an increase of oligodendroglial marker-positivity after a 1- and 3-day MSC-CM treatment which was similar in white and grey matter 4 days after transplantation (Figure 4.6; see also Samper Agrelo et al., 2020). A direct comparison of grey and white matter within the control or MSC-CM pre-treated group revealed, except of a significant decrease in the number MBP/GFP-positive cells after a 1-day MSC-CM pre-stimulation (Figure 4.6D), no difference in differentiation capacity of pre-stimulated aNSCs between white and grey matter 4 days post transplantation into the mouse brain.

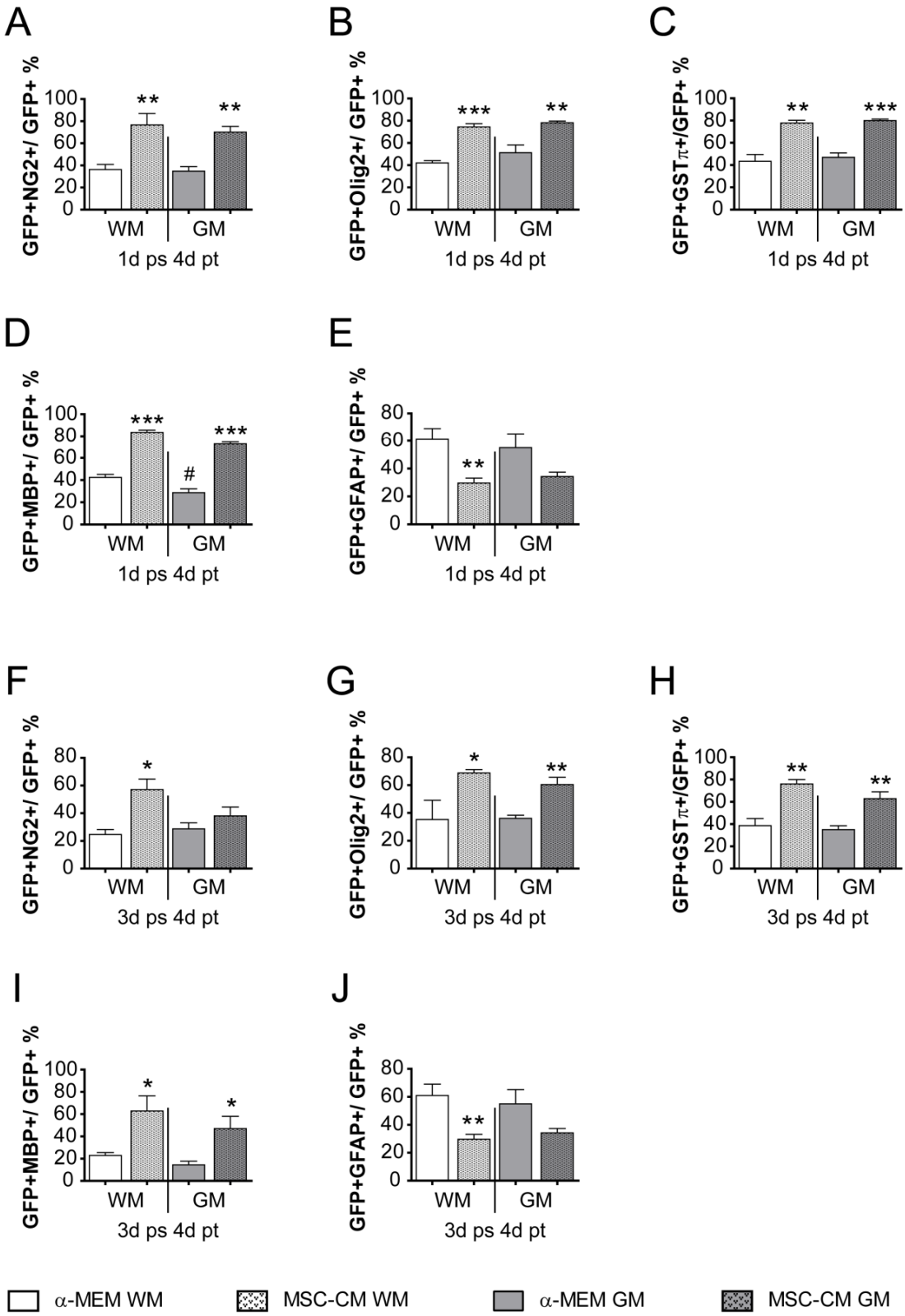

Figure 4.6: Endogenous signals of grey and white matter have no impact on the differentiation capacity of MSC-CM pre-stimulated aNSCs within the mouse brain after 4 days

(**A-J**) Quantification of transplanted aNSCs into the grey and white matter expressing oligodendroglial markers revealed a significantly increased number of cells expressing NG2 (**A, F**), Olig2 (**B, G**), GSTπ (**C, H**) and MBP (**D, I**) and a decreased number of GFAP-positive cells (**E, J**) after a 1 (**A-E**) or 3 day (**F-J**) pre-stimulation with MSC-CM at 4 days pt. No significant differences were found comparing grey and white matter transplantations of

control or MSC-CM treated NSCs independent of pre-stimulation or post transplantation time point, exception MBP. Here, a significant decrease of MBP/GFP-positive cells after a 1-day MSC-CM pre-stimulation at 4d pt was observed (**D**). For statistical analysis, a two-way ANOVA with Bonferroni posttest was used: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, MSC-CM compared to the respective α-MEM control (1 or 3 day pre-stimulation); # $p \leq 0.05$, compared white to grey matter (α-MEM or MSC-CM). Data are shown as mean values ± SEM derived from n=3-5 animals. Adapted from Figure S3: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

In addition to the 4 days post transplantation time-point, the differentiation capacity of MSC-CM pre-treated cells in the grey and white matter of the brain was further compared 7 days post transplantation. Quantifications of control and MSC-CM pre-treated aNSCs revealed an increased number of NG2-, Olig2-, GSTπ- and MBP-positive cells with a 1- and 3-day MSC-CM pre-stimulation 7 days after transplantation (Figure 4.7A-D, F-I). Similar to the 4-day transplantation, a reduction of GFAP-positive cells was observed after a 1- and 3-day MSC-CM stimulation 7 days post transplantation into the intact mouse brain (Figure 4.7E, J). Comparison of grey and white matter revealed no significant difference between these regions independent of control or MSC-CM pre-stimulation and duration (Samper Agrelo et al., 2020).

In summary, it could be demonstrated that the differentiation capacity of MSC-CM pre-stimulated aNSCs was not altered by a grey and white matter localization after transplantation into the mouse brain.

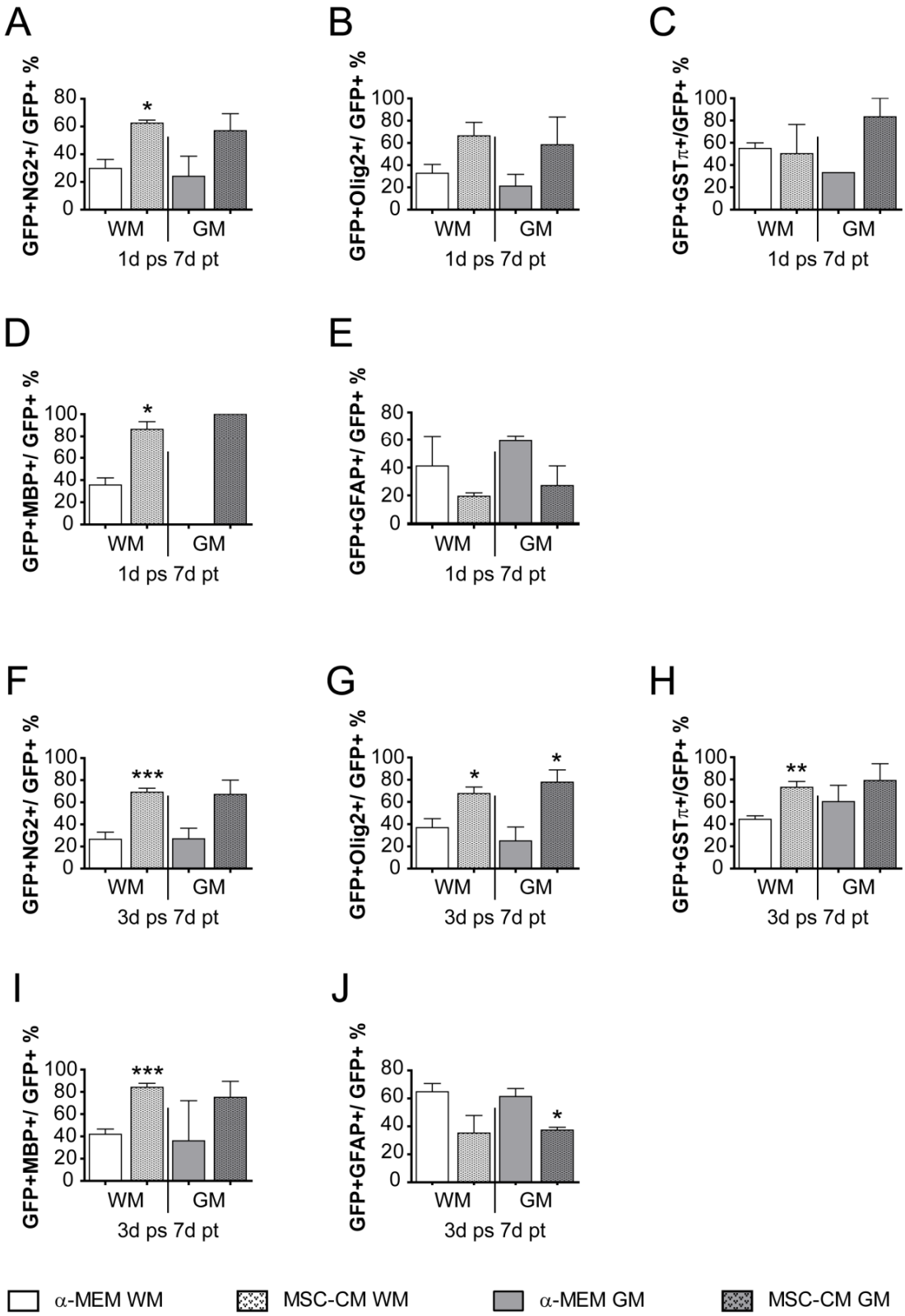

Figure 4.7: Endogenous signals of grey and white matter have no impact on the differentiation capacity of MSC-CM pre-stimulated aNSCs within the mouse brain at 7 days post transplantation

(**A-J**) Evaluation of GFP-positive cells 7 days after transplantation into the grey and white matter of the mouse brain revealed an increased number of cells expressing NG2 (**A, F**), Olig2 (**B, G**), GSTπ (**C, H**) and MBP (**D, I**) and a decreased number of GFAP-positive cells (**E, J**) after a 1 (**A-E**) or 3 day (**F-J**) pre-stimulation with MSC-CM. No significant differences were found comparing grey and white matter direct within control or MSC-CM independent of

pre-stimulation or post transplantation time point. For statistical analysis, a two-way ANOVA with Bonferroni posttest was used: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, MSC-CM compared to the respective α-MEM control (1 or 3 day pre-stimulation). Data are shown as mean values ± SEM derived from n=3-4 animals. Adapted from Figure S3: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.2 Limited survival rate of aNSCs after transplantation into the brain

To evaluate long-term survival and tissue integration, aNSCs were transplanted into the mouse brain and analyzed at 14 days post transplantation (unpublished data). Out of a total of 12 animals in the 3-day pre-stimulation group, GFP-positive aNSCs were only detected in 3 animals showing a dramatic decreased survival rate. A similar decrease in the survival rate of GFP-positive cells was found after 1-day pre-stimulation of aNSCs. Here, GFP-positive cells were only found in 2 out of 8 animals (data not shown). Due to the fact that in general too low numbers of GFP-positive cells were detected, it was not possible to investigate their differentiation capacity at 14 days post transplantation.

To evaluate whether the observed cell loss was caused by the xenogeneic transplantation paradigm (rat derived cells into the mouse brain), 3-day pre-stimulated rat aNSCs were transplanted into the intact rat brain (corpus callosum and cortex) and analyzed at 14 days post transplantation (unpublished data). Transplantation into the intact brain of 10-12 weeks old Wistar rat (n=1) revealed a decreased number of GFP-positive cells pre-treated with MSC-CM compared to control α-MEM (Figure 4.8A). At 14 days post transplantation, MSC-CM pre-stimulated aNSCs showed the same differentiation capacity as MSC-CM stimulated cells at 4 or 7 days after transplantation into the mouse brain (Figure 4.8B'-E'). The mature oligodendrocyte makers GSTπ and MBP revealed a 40% increase in the number of MSC-CM pre-stimulated cells (Figure 4.8C',D') whereas GFAP-positive cells were reduced (Figure 4.8E'). These data demonstrate that the long-term survival of transplanted cells was generally reduced in the rodent brain. Nevertheless, transplantation of MSC-CM stimulated aNSCs revealed in general a better survival in the rat brain compared to the mouse brain.

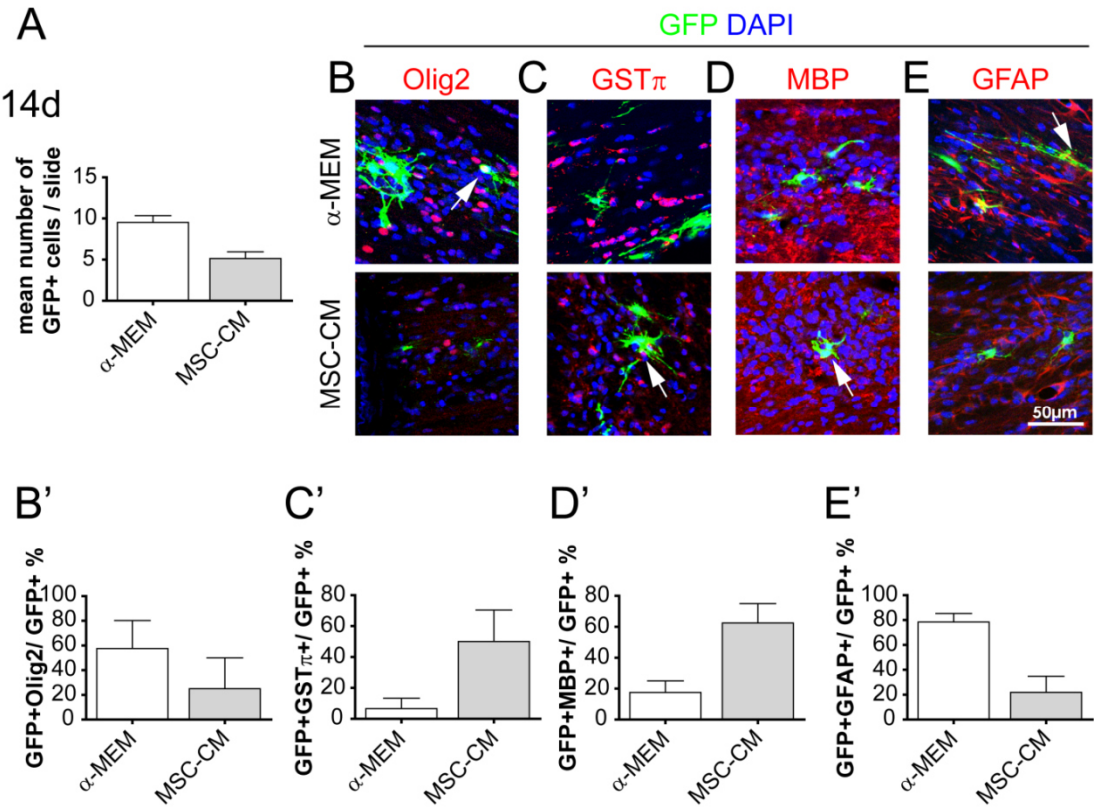

Figure 4.8: Reduced survival rate of MSC-CM pre-stimulated aNSCs at 14 days post transplantation into the rat brain

(**A**) Quantitative analysis of the mean number of GFP-positive cells per slide (2 slices per slide) pre-treated for 3 days with MSC-CM or control α-MEM 14 days post transplantation into the rat brain. Data are shown as mean values ±SEM (representing all analyzed GFP-positive cells per slide per staining) derived from n=1 animal per condition. (**B-E**) Representative images of GFP-positive cells expressing Olig2 (**B**), GSTπ (**C**), MBP (**D**) and GFAP (**E**) with corresponding graphs depicted in (**B'-E'**). Exemplary transplantation analysis revealed that 14 days after transplantation MSC-CM pre-stimulated aNSCs has the same differentiation capacity as after 4 or 7 days. Data are shown as mean values ±SEM (representing analyzed slices per marker) derived from n=1 animal per condition.

4.3 aNSC survival and differentiation after transplantation into the intact rat spinal cord

Due to the fact that stimulated aNSCs only survived a short time in the brain environment, MSC-CM pre-stimulated aNSCs were transplanted into the intact rat spinal cord to analyze their survival rate, tissue integration, cell fate, maturation stage and myelination ability in another CNS environment. Therefore, 3-day rat MSC-CM or α-MEM pre-stimulated GFP-positive aNSCs were implanted into the grey and white matter of adult rat spinal cords and the survival as well as differentiation and tissue integration was investigated at 14 and 28 days after transplantation (Samper Agrelo et al., 2020).

4.3.1 Prolonged aNSC survival rate after transplantation into the intact rat spinal cord

To evaluate the survival rate of transplanted cells, the number of GFP-positive cells per slide and animal at both time points post grafting into the intact rat spinal cord was evaluated. Quantification of transplanted cells revealed that 27 cells/slide/animal of α-MEM and 18 cells/slide/animal of MSC-CM pre-treated GFP-positive aNSCs survived for at least 2 weeks post transplantation, respectively. The number of GFP-positive cells decreased over time in both groups at 28 days post transplantation. The white matter environment seems to exert a general survival benefit on grafted cells compared to the grey matter (Figure 4.9B). Here, a significantly higher number of GFP-positive aNSCs pre-stimulated either with α-MEM (52 cells/slide/animal) or MSC-CM (36 cells/slide/animal) was detected compared to grey matter transplantations (α-MEM 23 cells/slide/animal; MSC-CM 6 cells/slide/animal; Figure 4.9B). In addition, 14 days post transplantation less MSC-CM stimulated cells compared to control α-MEM stimulated aNSCs were observed in both grey and white matter regions. However, at the later time point (28d) the survival rate was similar between α-MEM (9 cells/slide/animals) and MSC-CM (8 cells/slide/animals) pre-treated cells within the white matter while the number of α-MEM pre-stimulated cells decreased (3 cells/slide/animals) compared to MSC-CM (10 cells/slide/animals) in the grey matter (Figure 4.9C).

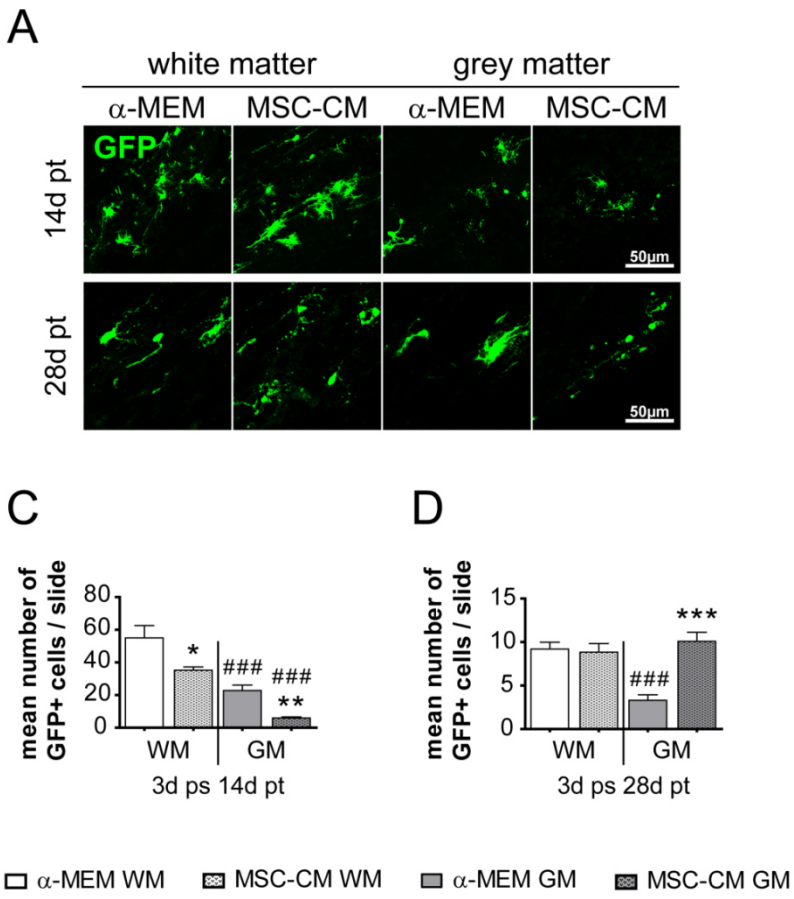

Figure 4.9: Survival rate of pre-stimulated rat aNSCs after spinal cord transplantations

(**A**) Immunohistochemical staining of GFP-positive transplanted cells (green) 14 and 28 days after transplantation into the grey and white matter of the intact rat spinal cord. Corresponding quantification of GFP-positive transplanted cells per slide (a mean of 3 sections per slide) and animal in grey and white matter at 14 (**B**) and 28 days (**C**). Statistical significance was calculated using a two-way ANOVA with Bonferroni posttest: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (for comparison between control α-MEM to MSC-CM) and ### $p \leq 0.001$ (for comparison between white and grey matter and respective transplantation paradigm). Data are shown as mean values ± SEM derived from n=4-6 animals, for more details see Table 3.14. Adapted from Figure 3: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.3.2 Differentiation capacity of MSC-CM pre-stimulated aNSCs after transplantation into the intact spinal cord

Since MSC-CM pre-stimulated NSCs transplanted into the intact rat spinal cord were still detectable at day 28 after transplantation their differentiation capacity was determined by means of immunohistochemical staining for different glial markers, which revealed an enhanced oligodendroglial cell fate of MSC-CM pre-stimulated aNSCs at 14 and 28 days post transplantation (Samper Agrelo et al., 2020). While

there was no impact of MSC-CM and α-MEM pre-stimulation on the number of Olig2-positive cells (Figure 4.10A,A'), a total of 67% at 14d and 76% at 28d of the MSC-CM pre-stimulated cells differentiated into GSTπ-positive and 70% at 14d and 59% at 28d differentiated into MBP-positive cells compared to the controls, respectively (GSTπ:α-MEM 37% 14d and 33% 28d; Figure 4.10B,B'; MBP: α-MEM 36% 14d and 21% 28d; Figure 4.10C,C'). In addition, the number of cells expressing oligodendroglial markers increased after MSC-CM treatment similar to the brain-transplanted cells whereas cells expressing the astroglial marker GFAP decreased. Here, MSC-CM stimulation resulted in a significant decrease of 25% of GFAP/GFP-positive cells compared to the control at 14 days post transplantation and 32% at 28 days pt (Figure 4.10D,D').

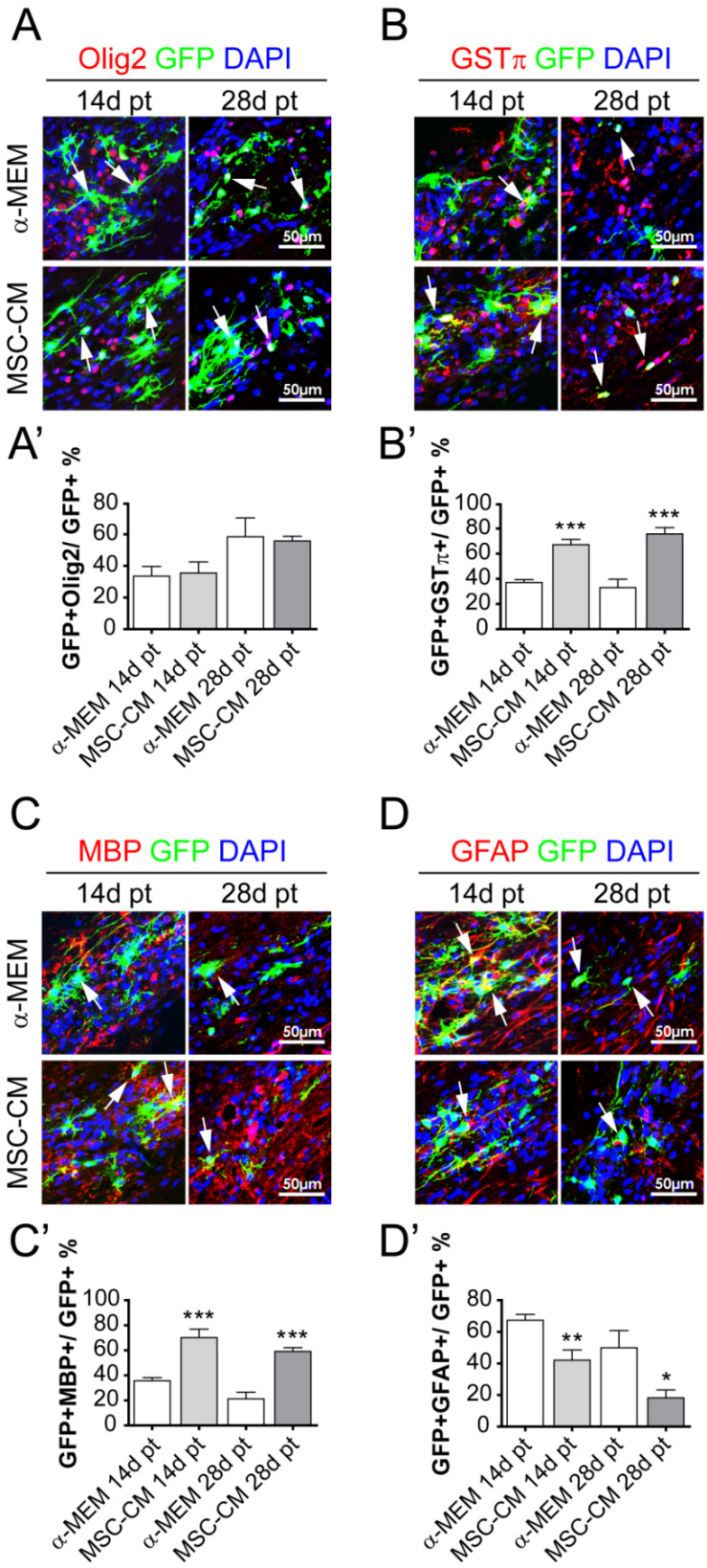

Figure 4.10: MSC-CM stimulation results in a pro-oligodendroglial differentiation of aNSCs transplanted into the spinal cord

(**A-D**) Representative pictures of GFP-positive cells (green) expressing the oligodendroglial marker Olig2 (**A**), GSTπ (**B**), MBP (**C**) and the astrocyte marker GFAP (**D**). (**A'-D'**) Corresponding quantitative evaluation (% of expressing cells over total number of GFP-expressing cells) of Olig2 (**A'**), GSTπ (**B'**), MBP (**C'**) and GFAP (**D'**) expression of

transplanted pre-treated aNSCs 14 and 28 days pt. Statistical significance was evaluated using a two-way ANOVA with Bonferroni posttest: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (for comparison between control and MSC-CM). Arrows point to GFP-positive cells (green) expressing the respective marker proteins (red). Data are shown as mean values ± SEM derived from n=4-6 animals. Adapted from Figure 4: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.3.2.1 Differentiation ability of MSC-CM stimulated aNSCs after transplantation into different spinal cord regions

To analyze whether different spinal cord regions have an impact on the differentiation behavior, MSC-CM and α-MEM pre-stimulated aNSCs were injected into the grey and white matter of rat spinal cords and analyzed at 14 and 28 days post transplantation. Independent of their grey or white matter localization MSC-CM pre-stimulated aNSCs showed the same pro-oligodendroglial differentiation ability as revealed by region-independent analysis at 14 and 28 days post transplantation (Figure 4.11). Of note, the mature oligodendrocyte and myelin markers GSTπ and MBP were significantly increased up to 40% compared to control treated cells independent of the transplantation region and time point (Figure 4.11B,C,F,G). In addition, no difference in the differentiation capacity was detectable between grey and white matter transplantations (Figure 4.11, also see Samper Agrelo et al., 2020). These results therefore show that the oligodendroglial differentiation capacity of MSC-CM pre-stimulated aNSCs is similar after transplantation into the spinal cord compared to the brain and that the CNS region has no influence on the establishment of pro-oligodendroglial properties.

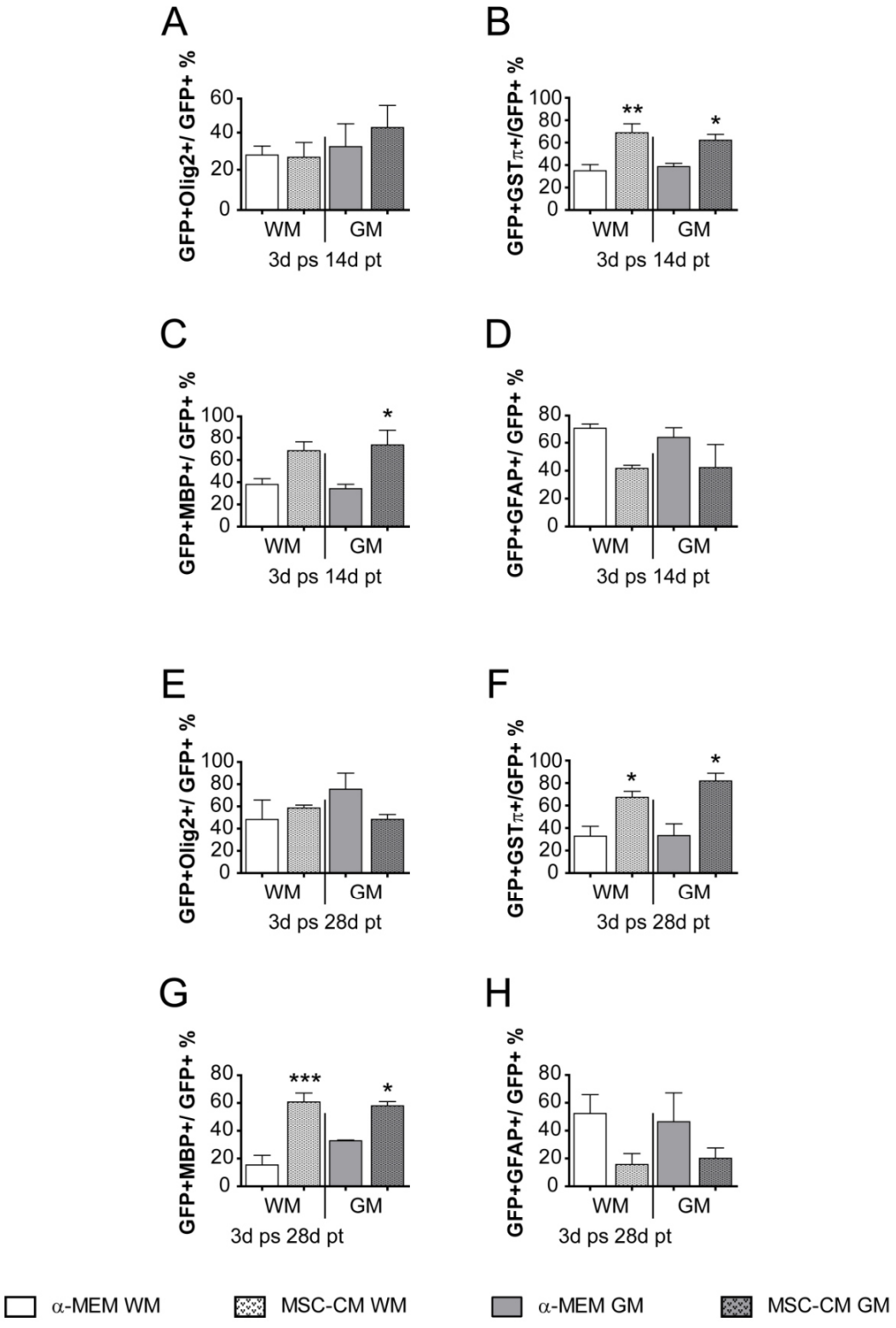

Figure 4.11: Region-independent differentiation capacity of MSC-CM pre-stimulated aNSCs localized in the grey and white matter of the spinal cord

(**A-H**) Distribution and quantification of GFP-positive MSC-CM or α-MEM pre-treated cells transplanted within the grey and white matter of the spinal cord after 14 and 28d. Grey and white matter of the spinal cord have no influence on the pro-oligodendroglial lineage differentiation of MSC-CM pre-stimulated aNSCs 14 and 28 days after injection. Statistical significance was evaluated using a two-way ANOVA with Bonferroni posttest: * $p \leq 0.05$, ** p

≤ 0.01, *** p ≤ 0.001 (for comparison between control to MSC-CM). Data are shown as mean values ± SEM derived from n=4-6 animals. Adapted from Figure 4: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.3.2.2 MSC-CM stimulated aNSCs do not express neuronal markers *in vivo*

In order to investigate whether transplanted aNSCs also showed a neuronal differentiation capacity after transplantation into the spinal cord, exemplary staining against the neuronal marker neuronal nuclei antigen (NeuN) was performed. No GFP/NeuN-positive cells were found after control (α-MEM) or MSC-CM pre-treatment after 14d (Figure 4.12, see also Samper Agrelo et al., 2020) indicating that the differentiation capacity into the neuronal lineage of aNSCs pre-treated with both media is rather low or even absent *in vivo*.

Taken together, these data demonstrate that MSC-CM pre-treated aNSCs survive for a long period after transplantation into the intact rat spinal cord and preferentially differentiate to mature MBP-expressing oligodendrocytes.

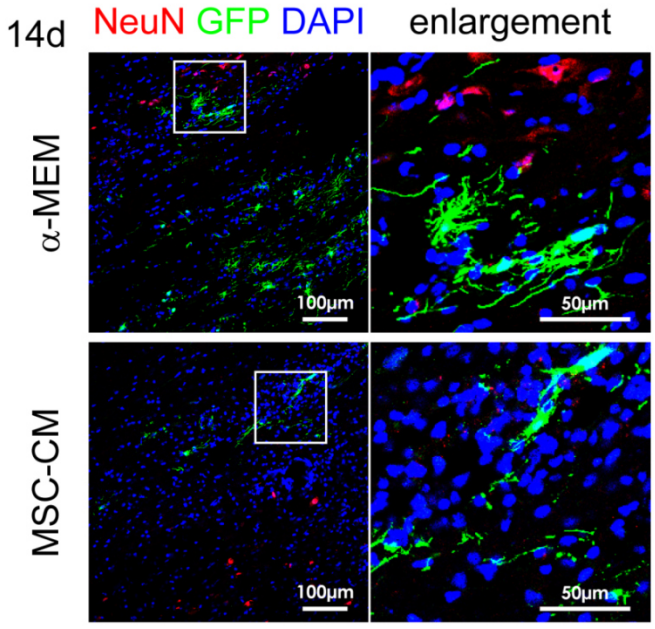

Figure 4.12: Absent neuronal differentiation capacity of transplanted aNSCs

Representative pictures of GFP-positive cells (green) showing no co-localization with NeuN (red) after 14d pt within the rat spinal cord independent of the pre-stimulation. Exemplary analysis of n=4-5 animals per group. Adapted from Figure S4: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.4 Myelination capacity of MSC-CM pre-stimulated aNSCs after transplantation into the intact spinal cord

The data gathered so far indicated that MSC-CM pre-treated transplanted aNSCs preferentially differentiate into MBP-expressing oligodendroglial cells. To analyze the functionality of these MBP-expressing cells and to reveal whether functional oligodendrocytes were generated, co-staining of 14d transplanted spinal cord slices with neuronal and paranodal markers were performed. Such structural analysis of myelin expressing transplanted cells was carried out by immunoelectron microscopy.

4.4.1 MSC-CM pre-stimulated aNSCs end in Caspr-positive paranodal regions

Immunohistochemical staining of transplanted MSC-CM pre-stimulated aNSCs revealed a co-localization of MBP/GFP-positive processes extending parallel to neurofilament-positive axons with α-MEM and MSC-CM pre-stimulation (Figure 4.13A-A''). While MBP-positive processes lying parallel to neurofilament positive axons were detected in transplanted aNSCs following both pre-treatment conditions (Figure 4.13A',A''), triple staining for GFP, MBP and the paranodal marker Caspr (Samanta et al., 2015; Koutsoudaki et al., 2016) revealed that only after MSC-CM pre-stimulation MBP/GFP-positive cells ending in Caspr-positive paranodal regions can be detected (Figure 4.13B,B''). These results indicate that MSC-secreted factors indeed contribute to the generation of myelinated segments, paranodal structures and hence fully matured oligodendrocytes *in vivo* (Samper Agrelo et al., 2020).

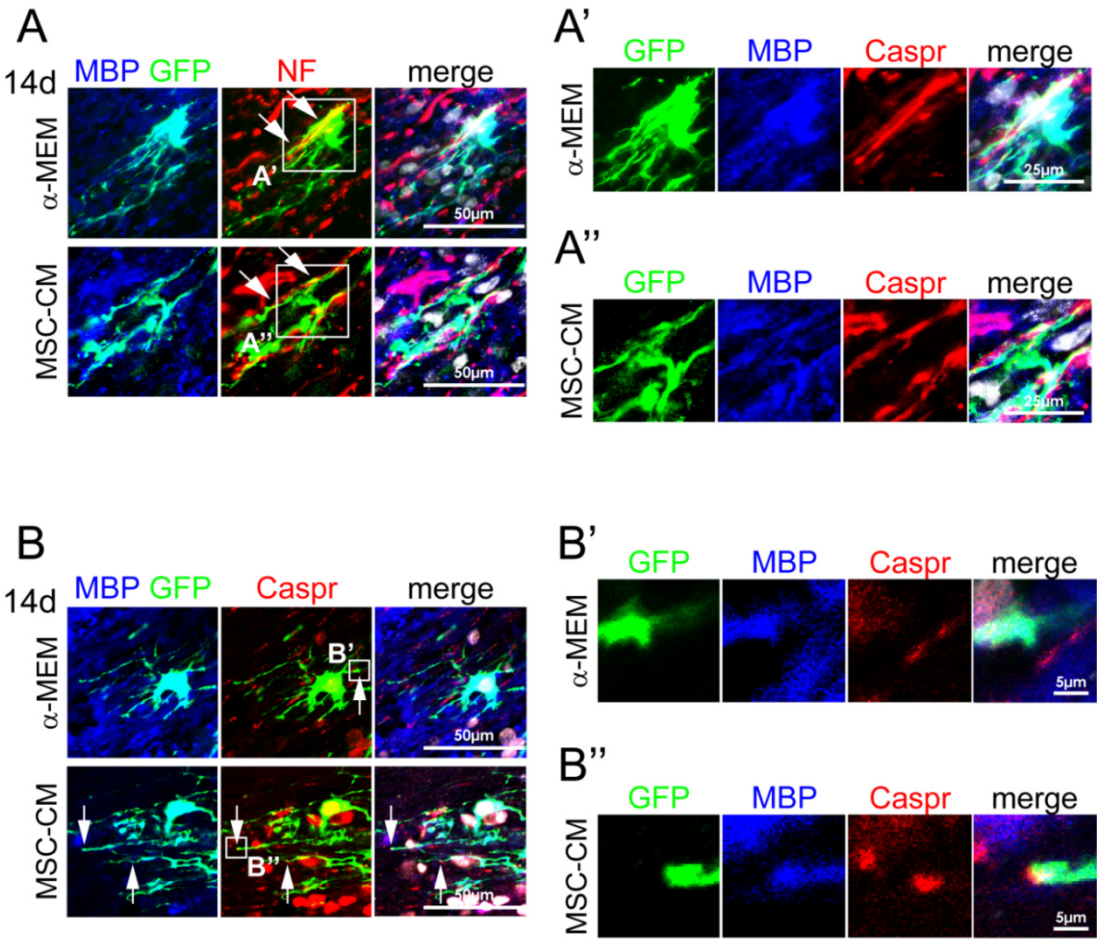

Figure 4.13: MSC-CM pre-stimulated aNSC are co-localized with marker for paranodal structures

(**A**) Exemplary immunohistochemical staining of MBP/GFP/neurofilament of spinal cord sections at 14 days post transplantation for α-MEM and MSC-CM pre-treatment. Transplanted cells aligned with neurofilament (NF)-positive axons (arrows) after both pre-treatments. Higher magnifications are shown for α-MEM in (**A'**) and MSC-CM in (**A''**). (**B**) Representative images of MBP/GFP/Caspr stained spinal cord sections 14d pt. MBP/GFP-positive processes of MSC-CM pre-treated and transplanted cells end in Caspr-positive paranodes (arrows). Nuclei are depicted in white. Higher magnifications are shown for α-MEM in (**B'**) and MSC-CM in (**B''**). Exemplary analysis of n=4-5 animals per group. Adapted from Figure 5: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.4.2 Ultrastructural analysis of MSC-CM pre-stimulated transplanted aNSCs show myelinated axonal segments

Prior immunohistochemical staining revealed that MSC-CM stimulated aNSCs are able to generate myelinated segments and to form paranodal structures after transplantation into the rat spinal cord. These findings were underlined by the ultrastructural analysis of MSC-CM pre-stimulated transplanted cells using

immunoelectron microscopy. Staining and immunoelectron microscopy was performed by our cooperation partners Dr. Janos Groh and Prof. Rudolf Martini from the Department of Neurology, Würzburg.

GFP-positive cells were identified in the spinal cord using a diaminobenzidine (DAB) staining (Figure 4.14A,B). Afterwards, transplanted cells were identified by chromogenic anti-GFP immunohistochemistry for immunoelectron microscopy resulting in dark precipitates. GFP-negative oligodendrocytes were also negative for dark precipitates (Figure 4.14C) whereas transplanted cells were found to form myelin sheaths around axons in the spinal cord (Figure 4.14D). Furthermore, GFP-immunoreactive signals were found within paranodal loops (Figure 4.14E). These data demonstrate that MSC-CM pre-stimulated aNSCs are able to form myelinated structures *in vivo* after transplantation (Samper Agrelo et al., 2020).

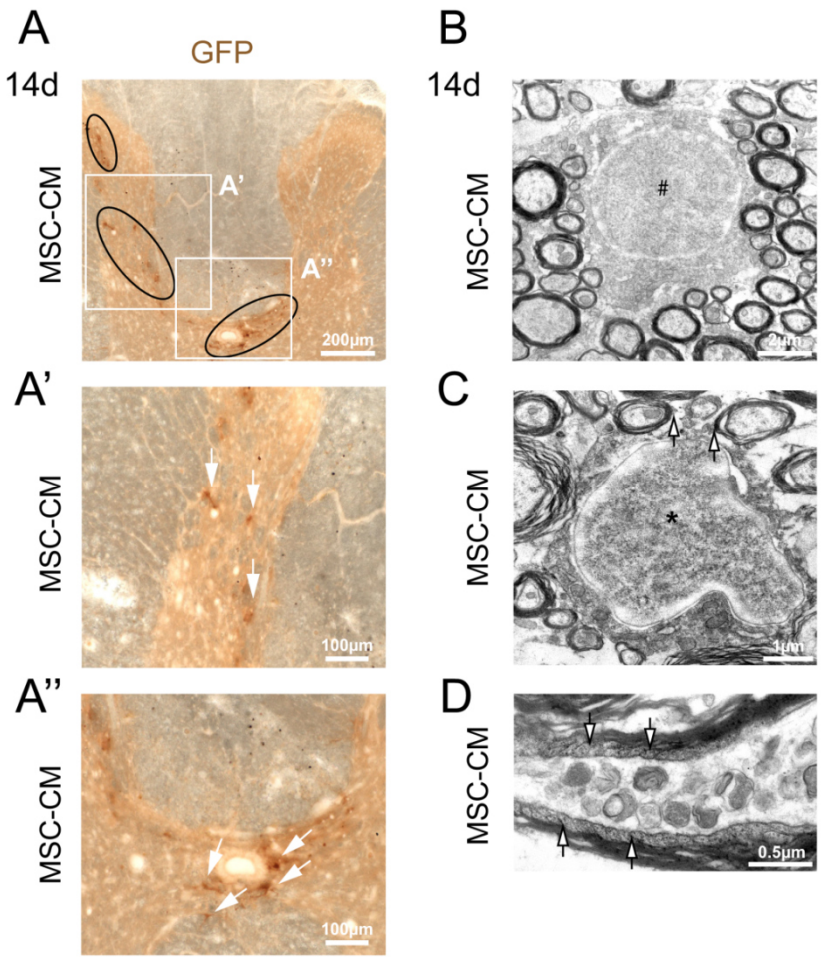

Figure 4.14: MSC-CM pre-stimulated aNSC are able form myelinated segments

(**A**) Immunohistochemical staining of GFP/DAB-positive cells in spinal cord sections at 14 days post transplantation for MSC-CM pre-treatment. Dark brown precipitates represent GFP-positive cells (black ovals). Higher magnifications are depicted in (**A'**) and (**A''**). Arrows point to GFP-positive transplanted cells. (**B**) Representative immunoelectron micrograph of a

GFP-negative oligodendrocytes (hash: nucleus) surrounded by myelinated axons. (**C**) Representative immunoelectron micrograph of a GFP-positive cell (asterisk: nucleus) 14d pt with chromogenic GFP-immunoreactivity (dark precipitate) myelinated processes (arrows). (**C''**) GFP-positive signals were also found in the cytoplasm of paranodal loops (arrows). Exemplary analysis of n=2 animals. Adapted from Figure 5: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.5 Secretome analysis of mesenchymal stem cell-conditioned medium

Based on the above-mentioned findings that MSC-secreted factors exert a strong pro-oligodendroglial impact on aNSCs in cell culture and *in vivo* the question arose which factors are responsible for these effects.

Although already several analyses of MSC-secreted components have been published, most of them dealing with the immunomodulatory or anti-inflammatory properties of MSCs (Maffioli et al., 2017; Spitzhorn et al., 2017; Teixeira et al., 2017; Baberg et al., 2019; Jungbluth et al., 2019; Timaner et al., 2020), none of them specifically addressed the pro-oligodendroglial properties of the MSC secretome. To answer the question regarding proteins responsible for the pro-oligodendroglial MSC-CM mediated effect, a mass spectrometry based secretome analysis was performed.

4.5.1 Serum free MSC-CM revealed pro-oligodendroglial properties

In order to be able to carry out a secretome analysis interfering proteins derived from serum must be excluded. To this end MSCs were conditioned in a serum-free N2-medium according to previously published protocols (Schira et al., 2015; Schira-Heinen et al., 2019; Schira et al., 2019). In a next step the differentiation behavior of serum-free MSC-CM stimulated aNSCs was analyzed after 3 days using two different N2-based MSC-CMs (derived from different MSC-CM passages), control N2 medium and “normal” rat MSC-CM. This analysis revealed that stimulation with MSC-CM and both N2-based MSC-CMs led to 12-15% CNPase-positive cells whereas only 1.3 % CNPase-positive cells were detectable after incubation with N2 control medium (Figure 4.15A,A’). Quantifications of GFAP-positive cells showed a strong increase of GFAP-positive cells (59%) after 3-day MSC-CM stimulation compared to N2 control (0%; Figure 4.15B’). While normal MSC-CM induced a strong GFAP-induction after 3 days, serum-free N2 MSC-CMs led only to a slight increase of 8% GFAP-positive

cells compared to N2 control medium. This indicated that under serum free conditions at least pro-oligodendroglial factors were correctly released and that therefore the N2-based conditioned medium was indeed suitable for a secretome analysis.

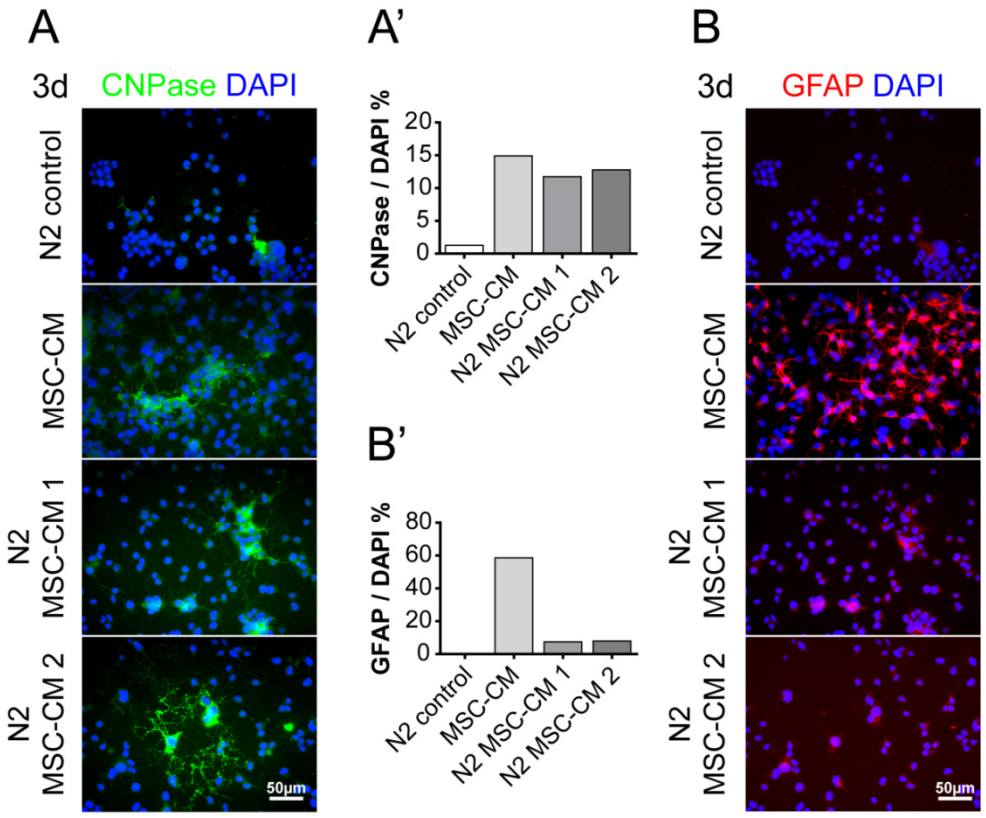

Figure 4.15: Serum-free MSC-CM has the same potential to promote oligodendroglial differentiation as normal MSC-CM

(**A**) Representative pictures of CNPase-positive cells after a 3-day stimulation with MSC-CM, N2 MSC-CM or N2 control medium. (**A'**) Quantitative evaluation of the number of CNPase-positive cells per DAPI revealed a similar increase of CNPase-expressing cells after stimulation with MSC-CM and both N2-based MSC-CMs. (**C**) Anti-GFAP immunofluorescent staining 3 days after stimulation with N2 control, rat MSC-CM and two N2-based MSC-CMs revealed a strong increase (**B'**) of GFAP-positive cells after MSC-CM treatment and a slide increase after N2-based MSC-CM stimulation compared to control. Data are shown as mean values ± SEM (representing 10 pictures per condition) derived from n=1 experiment.

4.5.2 Comparative secretome analysis of mesenchymal stem cell-conditioned medium revealed 152 enriched proteins in the secretome

Several secretome analyses of MSC-secreted components dealt with their immunomodulatory or anti-inflammatory properties (Maffioli et al., 2017; Spitzhorn et al., 2017; Teixeira et al., 2017; Baberg et al., 2019; Jungbluth et al., 2019). However, none these studies addressed the pro-oligodendroglial properties of the MSCs. In order to identify active oligodendrogenesis modulating components in the MSC

secretome and to receive hints regarding the underlying regulatory mechanisms a mass spectrometry based secretome analysis was performed in cooperation with Prof. Kai Stühler (Molecular Proteomics Laboratory, BMFZ, Heinrich-Heine-University, Düsseldorf). For mass spectrometry analysis, four replicates of MSCs were incubation with serum-free N2-medium for 48h and analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS; Figure 4.16A). In order to identify *bona fide* secreted proteins, the abundances of proteins identified in the secretome were compared to the proteins abundances in the corresponding proteome according to Grube et al., (2018). The representative heatmap of all investigated samples revealed that significantly less proteins were identified in the secretome compared to the corresponding proteome, but the respective replicates were homogenous indicating a good reproducibility (Figure 4.16B). A total number of 691 proteins was identified in the MSC-CM of which 152 proteins were detected as *bona fide* secreted proteins due to the comparison to the cellular proteome (Figure 4.16C; see also Table 10.1, annex; raw data are available at PRIDE database with dataset identifier PXD018231; also see Samper Agrelo et al., 2020). From these *bona fide* secreted proteins, 85% were predicted to have a signal peptide, thus these proteins were likely secreted by the classical secretory pathway such as for example the glia-derived nexin (Serpine2) or vesicant core protein (Vcan). In addition, 7% exhibited a transmembrane domain indicating a localization in the cell membrane. Thus, these proteins could be shedded extracellularly such as for example semaphorin 3D (Sema3d) and a disintegrin and metalloproteinase with thrombospondin motifs 1 (Adamts1). Using the Outcyte prediction tool (Zhao et al., 2019) to predict classically as well as unconventionally secreted proteins revealed that 1% of the proteins such as for example platelet-derived growth factor C (Pdgfc) or syntenin-1 (Sdcbp) can be assumed to be non-conventionally secreted (Figure 4.16D).

Proteins enriched in the MSC-derived secretome were associated with extracellular components such as the laminin complex [enrichment factor (EF) 11.4, *p*-value 6.50 x 10^{-4}], the proteinaceous extracellular matrix (EF 10.3, *p*-value 5.6 x 10^{-18}), extracellular matrix (EF 8.9, *p*-value 3.3 x 10^{-30}) and extracellular space (EF 7.2, *p*-value 8.8 x 10^{-25}) as revealed by categorical enrichment analysis. Additional analysis revealed that *bona fide* secreted proteins were under-represented in categories related to intracellular compartments such as the nucleus (EF -3.0, *p*-value 4.7 x 10^{-}

^{6}), the macromolecular complex (EF -4.3, *p*-value 1.2 x 10^{-16}) and the ribonucleoprotein complex (EF -12.0, *p*-value 7.8 x 10^{-8}) (Figure 4.16E). Further association to biological processes related to extracellular compartments such as the extracellular matrix organization (EF 7.9, *p*-value 1.0 x 10^{-15}), cell adhesion (EF 5.5, *p*-value 5.9 x 10^{-18}) and locomotion (EF 2.7, *p*-value 7.4 x 10^{-4}; Figure 4.16F) was found whereas proteins related to intracellular processes such catabolic processes (EF -5.2, *p*-value 1.4 x 10^{-6}) and translation (EF -12, *p*-value 2.6 x 10^{-3}) were under-represented (Figure 4.16F).

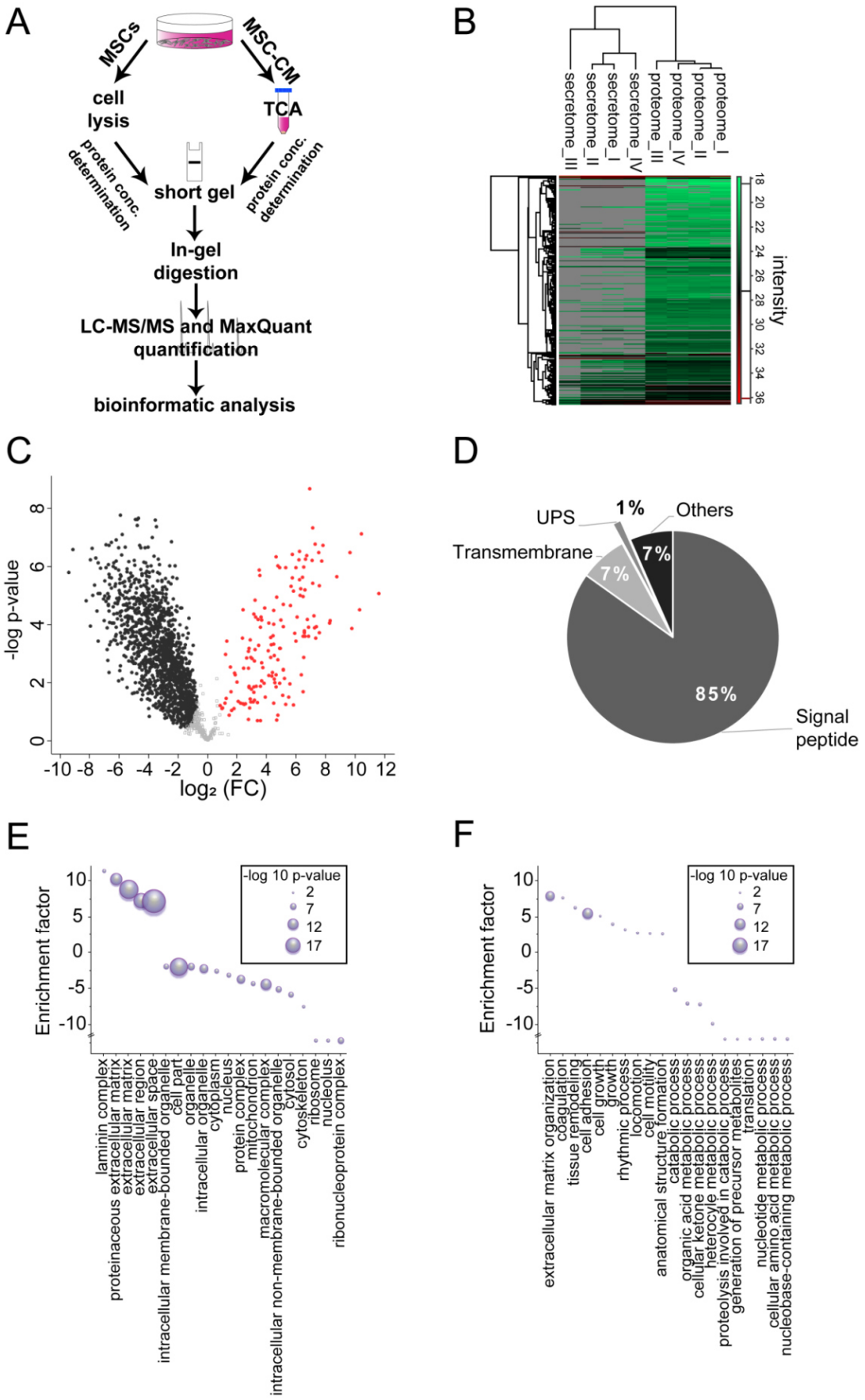

Figure 4.16: Mesenchymal stem cell secretome analysis

(**A**) Schematic workflow for the preparation of MSC-derived proteomes and secretomes for quantitative LC-MS/MS analysis. The heatmap depicted in (**B**) revealed that less proteins

were identified in the secretome compared to the proteome with respective replicates being similar to each other. (**C**) Volcano plot showing the 152 proteins detected as *bona fide* secreted in the MSC-derived secretome (red dots on right side) and proteins enriched in the MSC proteome. (**D**) The bona fide secreted proteins included several proteins predicted to have a signal peptide (85%), 7% exhibited a transmembrane domain and only 1% were unconventionally secreted (UPS). (**E**) GO enrichment analysis of cellular components showed that the secreted proteins were associated with the extracellular space and matrix localization. (**F**) Regarding biological processes, GO enrichment analysis revealed an association with extracellular matrix organization and cell adhesion (5% FDR). Replicates used for experiments n=4. Adapted from Figure 6: (Samper Agrelo et al., 2020) Secretome analysis of mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

According to literature, several proteins identified in the MSC secretome are known to either exert a positive or a negative impact on oligodendrocyte differentiation or on myelination (Table 4.1; also see Samper Agrelo et al., 2020) including growth factors as well as extracellular matrix proteins. Extracellular matrix proteins with a known positive impact on oligodendrogenesis are for example TIMP-1, dystroglycan (Dag1), laminin subunit beta-2 (Lamb2), osteopontin (Spp1) or the BMP-associated protein chordin (Chrd). According to the literature, also extracellular matrix proteins with a putative negative impact on oligodendrocyte differentiation or myelination were identified such as aggrecan (ACN), TNC, thrombospondin 1 (THBS1) or bone morphogenetic protein-1 (BMP-1) as well as growth factors such as connective tissue growth factor (CTGF), known to negatively regulate myelination, and insulin-like growth factor-binding proteins (IGFBP2 and IGFBP7).

Table 4.1: Mesenchymal stem cell secreted proteins previously associated with oligodendroglial lineage regulation

ID	Gene name	Oligodendroglial lineage promoting factor	Description	Secretion via / localization	Ref.
P07897	Acn	Aggrecan core protein	inhibits oligodendrocyte myelination	SP[1]	(Keough et al., 2016)
F1M798	BMP1	Metalloendopeptidase / bone morphogenetic protein 1	inhibits oligodendrogenesis	SP	(See et al., 2004)

A0A140TA94	Chrd	Chordin	promotes oligodendrogenesis from SVZ stem cells *in vitro* and *in vivo*	SP	(Jablonska et al., 2010)
Q9R1E9	Ctgf	Connective tissue growth factor	negatively regulates myelination	SP	(Ercan et al., 2017)
F1M8K0	Dag1	Dystroglycan 1	intracellular portion of cleaved dag promotes OPC proliferation / regulates myelin membrane production, growth, or stability.	SP	(Colognato et al., 2007; Leiton et al., 2015)
A0A096P6L8	Fn	Fibronectin	maintenance and proliferation of OPC	SP	(Lourenco et al., 2016)
Q63772	Gas6	Growth arrest-specific protein 6	protects oligodendrocytes from TNFα induced apoptosis	SP	(Shankar et al., 2006)
P12843	Igfbp2	Insulin-like growth factor-binding protein 2	negative effector of oligodendrocyte survival & differentiation	SP	(Kühl et al., 2002)
F1M9B2	Igfbp7	Insulin-like growth factor-binding protein 7	inhibits the differentiation of oligodendrocyte precursor cells via regulation of Wnt/β-Catenin signaling	SP	(Li et al., 2018)
P12843	Lamb2	Laminin subunit beta-2	extracellular matrix proteins known to be important for myelination	SP	(Relucio et al., 2012; Kim et al., 2014)

P30120	TIMP-1	tissue inhibitor of metalloproteinase 1	promotes oligodendrocyte differentiation and CNS myelination	SP	(Moore et al., 2011; Nicaise et al., 2019)
P08721	Spp1	Osteopontin	stimulates MBP synthesis and myelin sheath formation *in vitro*	SP	(Selvaraju et al., 2004)
F7EPE0	Psap	Sulfated glycoprotein 1 / Prosaposin	known as a myelinotrophic factor protecting myelinating glial cells	SP	(Hiraiwa et al., 1999; Meyer et al., 2014)
Q9JI92	Sdcbp	Syntenin-1	plays a role in OPC migration	UPS[2]	(Chatterjee et al., 2008)
M0R979	Thbs1	Thrombospondin 1	reduces oligodendrogenesis	SP	(Lu and Kipnis, 2010)
A0A0G2K1L0	Tnc	Tenascin c	inhibits OPC differentiation and myelination	SP	(Czopka et al., 2010)

[1]SP signal peptide; [2] UPS unconventional protein secretion

4.6 Protein candidates and their functionality

After identification MSC derived factors likely to have an impact on the oligodendroglial linage differentiation (Table 4.1), selected candidates were further investigated in cell culture. For this approach, specific antibody blocking assays were performed. MSC-CM as well as α-MEM were pre-treated with neutralizing antibodies and control IgG. Afterwards, aNSCs were treated with the aforementioned media for 3 days and subsequently analyzed regarding their differentiation potential via immunocytochemical staining. Two proteins with known influence to promote oligodendrogenesis and CNS myelination were chosen for this functional

investigation, the BMP antagonist chordin and TIMP-1 (Jablonska et al., 2010; Moore et al., 2011; Nicaise et al., 2019).

4.6.1 Neutralization of chordin did not alter the MSC-CM induced oligodendroglial differentiation of cultured aNSCs

Chordin was identified a bona fide secreted protein, acts as BMP antagonist and known to promote oligodendrogenesis from SVZ NSCs (Jablonska et al., 2010). A previous study demonstrated that chordin is induced in response to the suppression of the oligodendroglial inhibitor p57kip2 (Jadasz et al., 2012). For the present analysis, a chordin-blocking antibody was applied to α-MEM and MSC-CM one hour before aNSC stimulation. For control, normal media (without antibodies) as well as IgG isotope control were used. Quantitative analysis revealed that the number of CNPase-positive cells was significantly increased after a 3-day stimulation with MSC-CM, chordin neutralized MSC-CM and IgG isotope control treated MSC-CM compared to the corresponding α-MEM media (Figure 4.17A,C, unpublished data). However, no difference in the number of CNPase-positive cells were found between MSC-CM, chordin-neutralized MSC-CM or IgG isotope control treated MSC-CM (Figure 4.17C). Quantification of GFAP-positive cells revealed no significant difference between MSC-CM and α-MEM media independent of control, chordin-blocked and IgG isotope control media. Here, 50-90% of the cells were GFAP-positive (Figure 4.17D).

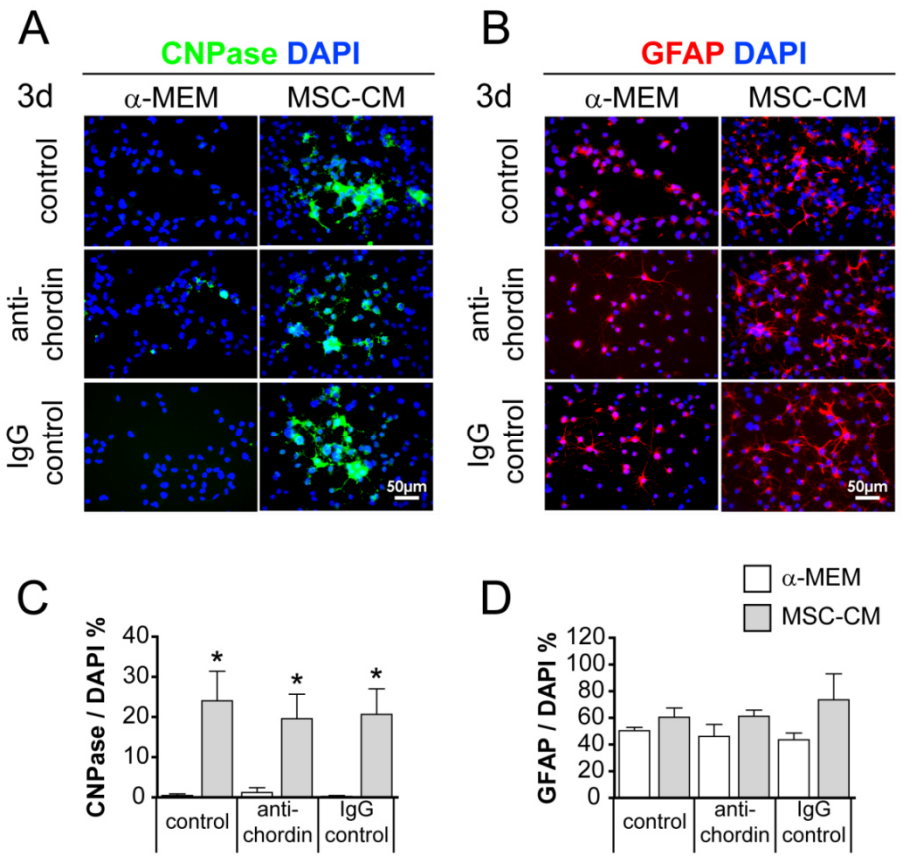

Figure 4.17: Blocking of chordin in MSC-CM did not affect its pro-oligodendroglial properties on cultured aNSCs

Immunohistochemical staining of CNPase- (**A**) and GFAP-positive (**B**) cells after a 3-day stimulation with α-MEM and MSC-CM (control) or either anti-chordin or IgG control. (**C**) Quantifications of CNPase-positive cells revealed an increase after all 3 MSC-CM media comparing to matching α-MEM media controls. No difference was found between the investigated MSC-CM conditions. (**D**) Analysis of GFAP-positive cells showed no difference between all 6 used media. Statistical significance was calculated using a two-way ANOVA with a Bonferroni posttest: * $p \leq 0.05$, ** $p \leq 0.01$. Data are shown as mean values ± SEM derived from n=3 experiments.

4.6.2 TIMP-1 blocking results in decrease of CNPase-positive cultured aNSCs

Next the influence of TIMP-1, a protein which was also found to be enriched in the MSC secretome, was tested. TIMP-1 is a tissue inhibitor of metalloproteinase 1 with known pro-oligodendroglial properties (Moore et al., 2011; Jiang et al., 2016; Nicaise et al., 2019). For blocking analysis, a TIMP-1 neutralizing antibody as well as corresponding IgG isotope control and no antibody control were incubated with MSC-CM for 1 hour before application on aNSCs for 3 days in culture. To exclude intrinsic TIMP-1 antibody effects this procedure was also done in parallel with non-conditioned α-MEM. All 6 media were incubated for 3 days on aNSCs and afterwards cells were evaluated for CNPase- and GFAP expression (Figure 4.18A,B; Samper Agrelo et al.,

2020). Incubation of control MSC-CM and IgG antibody control resulted in a significantly increased degree of CNPase-positive cells compared to the corresponding α-MEM treated cells. Untreated MSC-CM and IgG control showed no difference compared to each other. Blocking TIMP-1 in MSC-CM resulted in a significant reduction of CNPase-positive cells (2.8%) compared to untreated MSC-CM (11%) and IgG control (9%; Figure 4.18C). Furthermore, it was shown that neutralization of TIMP-1 resulted in a slight increase of GFAP-positive cells compared to normal MSC-CM and IgG control (Figure 4.18D). Again, MSC-CM and the IgG isotope control showed no differences compared to each other and showed the same degree of GFAP-positive cells compared to the corresponding α-MEM media (Figure 4.18D).

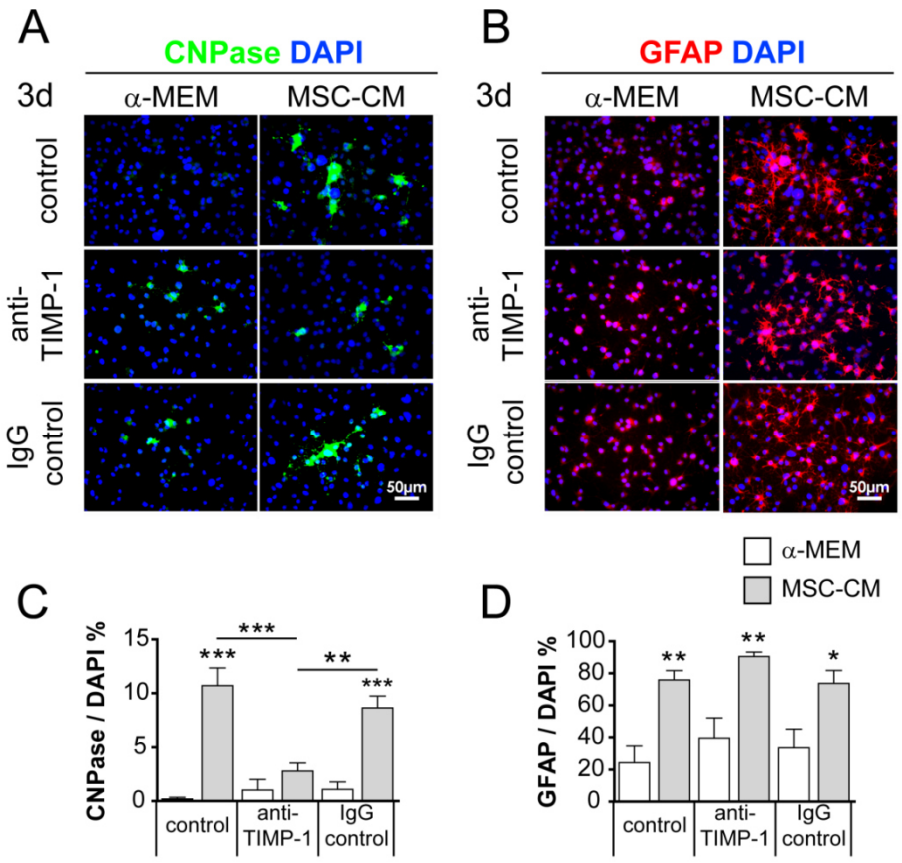

Figure 4.18: TIMP-1 neutralization in MSC-CM results in a reduced pro-oligodendroglial effect on cultured aNSCs

(**A-B**) Representative pictures of CNPase- and GFAP-positive cells. Cells were treated with either no antibody, anti-TIMP-1 or IgG isotope control pre-incubated MSC-CM or α-MEM. (**C**) Quantification of the number of CNPase-positive cells revealed a significant decrease of positive cells after neutralization of TIMP-1 in MSC-CM. (**D**) A slight increase of GFAP-positive cells was found using TIMP-1 blocked MSC-CM on aNSCs after 3 days. Statistical significance was calculated using a two-way ANOVA with a Bonferroni posttest: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data are shown as mean values ± SEM derived from n=3 experiments. Adapted from Figure 7: (Samper Agrelo et al., 2020) Secretome analysis of

mesenchymal stem cell factors fostering oligodendroglial differentiation of neural stem cells *in vivo*. Int. Journal. Mol. Sci. 21(12), 4350; https://doi.org/10.3390/ijms21124350. With permission from Multidisciplinary Digital Publishing Institute (MDPI, Basel, Switzerland).

4.7 Neutralization of TIMP-1 in MSC-CM reduces the oligodendroglial differentiation capacity *in vivo*

Due to the fact, that TIMP-1 neutralization led to a reduction of the aNSC oligodendroglial differentiation capacity, transplantations of TIMP-1 neutralized MSC-CM pre-stimulated aNSCs into the intact rat spinal cord were performed (unpublished data).

Prior transplantation into the spinal cord, neutralization of TIMP-1 and IgG control antibody in MSC-CM was performed as described above and aNSCs were pre-stimulated for 3 days. Pre-stimulated aNSCs were transplanted into the grey and white matter of the spinal cord at thoracic level eight (Th8) followed by immunohistochemical analysis at 14 days after transplantation. The total mean number of GFP-positive cells per slide per animal resulted in 29 cells for IgG control MSC-CM treated aNSCs and 11 cells for TIMP-1 neutralized MSC-CM pre-stimulated aNSCs revealing a slight decrease of GFP-positive aNSCs treated with TIMP-1 neutralized MSC-CM (Figure 4.19A). Oligodendroglial marker analysis showed a trend for decreased differentiation capacity after TIMP-1 neutralization since the number of GSTπ-positive cells was reduced compared to control (Figure 4.19B,B'). Approximately 65% of control treated GFP-positive cells were GSTπ-positive (Figure 4.19B,B') representing the same degree of GSTπ- positive cells as shown before with MSC-CM pre-stimulated aNSCs (see Figure 4.10B,B'). In addition, aNSCs pre-stimulated with TIMP-1-neutralized MSC-CM displayed a decreased number of MBP-positive cells compared to aNSCs stimulated with IgG control MSC-CM (Figure 4.19C,C'). Again, the number of MBP-positive cells after IgG control treatment reflected the number of MBP-positive cells using the normal MSC-CM (compare Figure 4.10C,C' to Figure 4.19C,C'). Analysis of the astrocyte marker GFAP revealed that TIMP1 neutralization led to a slightly higher number of GFP/GFAP-positive cells compared to IgG control cells 14 days post transplantation (Figure 4.19D,D') reflecting the data revealed in cell culture (Figure 4.18). Furthermore, aNSCs stimulated with the IgG control treated MSC-CM showed the same differentiation

capacity upon stimulation with normal MSC-CM (compare Figure 4.10D,D' to Figure 4.19D,D').

Since only two control animals could be considered for the above-mentioned quantification, statistical analysis could not be performed. However, these data reflect the cell culture experiments confirming that TIMP1 is an important component in MSC-CM to promote oligodendroglial differentiation of aNSCs.

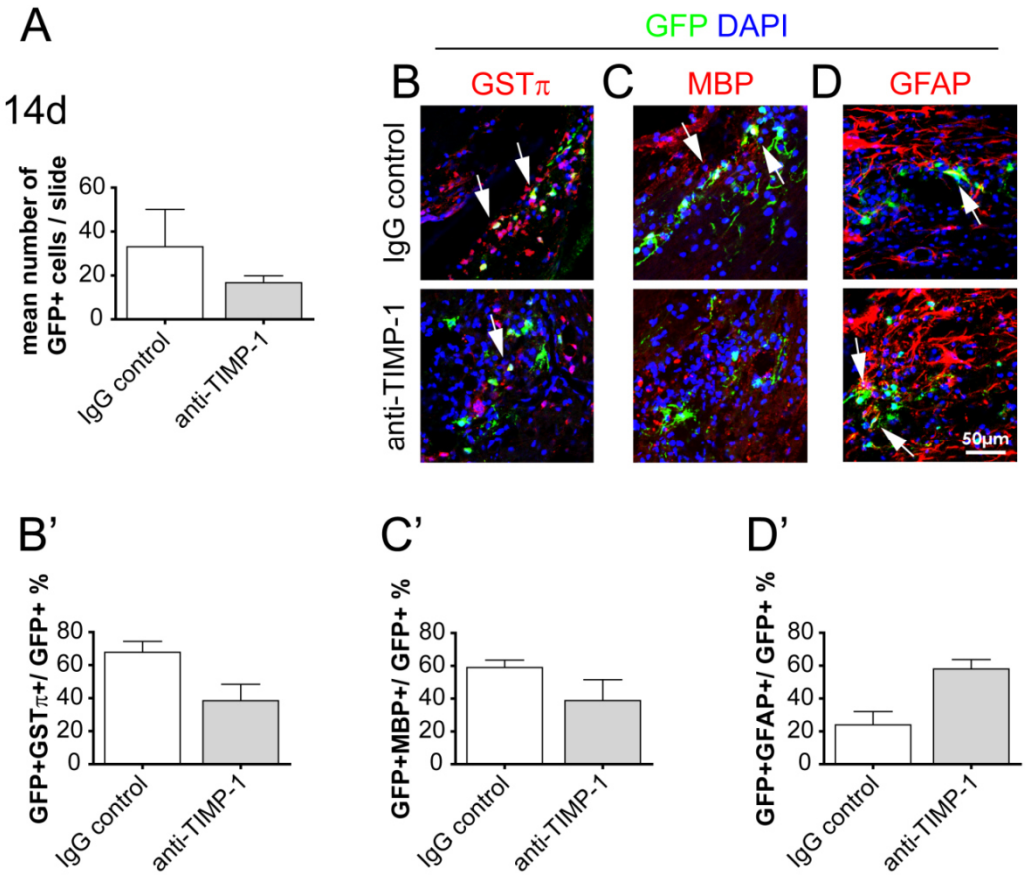

Figure 4.19: TIMP-1 MSC-CM neutralization results in reduced oligodendroglial differentiation of pre-stimulated aNSCs post transplantation into the spinal cord

(**A**) Quantitative analysis of the mean number of GFP-positive cells per slide (2 sections per slide) pre-treated for 3 days with TIMP-1 neutralized MSC-CM or IgG control treated MSC-CM 14 days post transplantation into the intact rat spinal cord. (**B-D**) Representative images of GFP-positive cells expressing GSTπ (**B**), MBP (**C**) and GFAP (**D**) with corresponding quantifications depicted in **B'-D'**. Transplantation analysis revealed that 14 days after transplantation TIMP-1 neutralized MSC-CM pre-stimulated aNSCs showed a slightly decreased number of oligodendroglial marker positive cells and an increase of GFAP-positive cells. Data are shown as mean values ± SEM derived from n=2-4 animals.

4.8 Human iPSCs derived MSC-CM promotes oligodendrogenesis of aNSCs in culture

The above described experiments clearly showed positive effects of MSC-CM secreted factors on the oligodendroglial differentiation capacity of aNSCs in culture and *in vivo* (Samper Agrelo et al., 2020). However, these data are all based on rodent cells and tissue. It is known that the positive impact of MSC-CM on oligodendroglial differentiation of NSCs is not restricted to rodents since fetal human MSC-CM has been shown to provide a similar oligodendroglial potential (Jadasz et al., 2018).

For future biomedical translation, MSC-CM derived from human iPSC derived cells could be an attractive option to replace bone marrow derived stem cells. To investigate whether MSCs derived from iPSCs also secrete pro-oligodendroglial factors, aNSCs were incubated with MSC-CM derived from iPSC-derived MSCs (iMSC-CM). The effect of rat MSC-CM, fetal human MSC-CM (hMSC-CM) and fetal human MSC derived iMSC-CM on aNSCs differentiation was compared at 3 and 7 days of MSC-CM incubation. Stimulation of aNSCs with MSC-derived factors from all MSC sources resulted in a significant increase of CNPase-positive cells culminating after 7 days with all three media (MSC-CM 25% at 3d, 47% at 7d; hMSC-CM 36% at 3d, 74% at 7d; iMSC-CM 24% at 3d, 73% at 7d, respectively) compared to α-MEM control medium (14% at 3d and 31% at 7d, respectively) (Figure 4.20A-A'', unpublished data). Concomitantly, 7 days after stimulation with MSC-CM, hMSC-CM and iMSC-CM, aNSCs significantly differentiated into MBP-positive cells (Figure 4.20B-B'', MSC-CM 14% at 3d, 54% at 7d; hMSC-CM 21% at 3d, 85% at 7d; iMSC-CM 9% at 3d; 62% at 7d, respectively) at an expense of GFAP-positive cells (Figure 4.20C-C'', MSC-CM 64% at 3d, 50% at 7d; hMSC-CM 48% at 3d, 25% at 7d; iMSC-CM 57% at 3d; 27% at 7d, respectively), indicating that beside MSCs derived from rat and human bone marrow also MSCs derived from induced pluripotent stem cells secrete trophic factors promoting the oligodendroglial lineage differentiation (unpublished data).

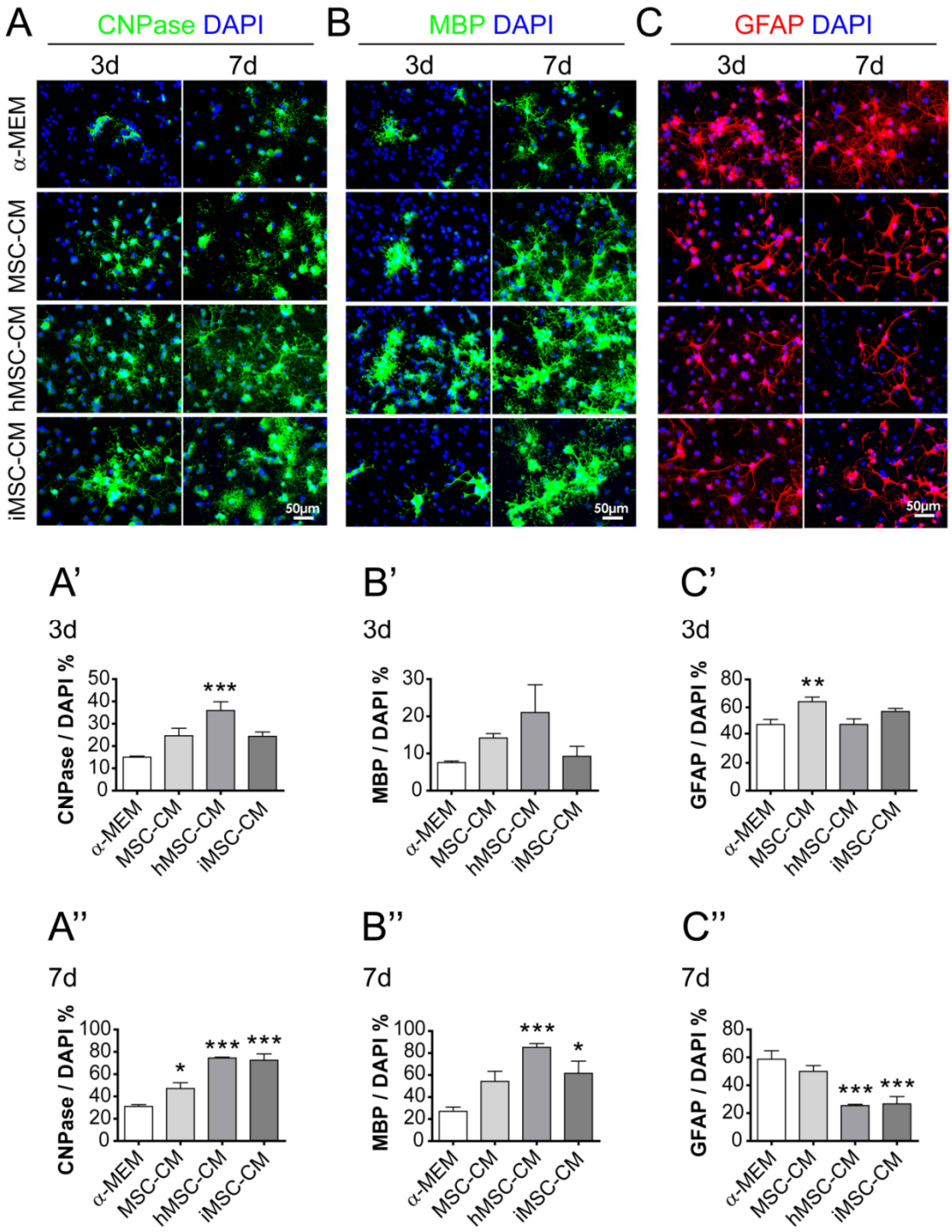

Figure 4.20: iMSC-CM has a similar potential to promote oligodendroglial lineage differentiation like human and rat MSC-CM

(**A**) Representative images and (**A'-A''**) quantification of CNPase- and (**B-B''**) MBP-positive cells relative to DAPI-positive nuclei revealing an increased number of oligodendroglial cells generated by all three conditioned media 3d and 7d after incubation. (**C**) Immunofluorescent staining revealed a significant increase of GFAP-positive cells 3d (**C'**) after stimulation followed by a decrease after 7d (**C''**). For statistical analysis a one-way ANOVA with Dunnett's multiple comparison test was performed with statistical significance threshold set as *p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001. Data are shown as mean values ± SEM derived from n=3 experiments.

5. Discussion

Multiple sclerosis is the most common demyelinating disease in which demyelination is counteracted by myelin repair activities based on recruitment and differentiation of resident progenitor- and stem cells leading to the generation of new myelinating oligodendrocytes (Franklin, 2002; Picard-Riera et al., 2002; Nait-Oumesmar et al., 2007; Nait-Oumesmar et al., 2008; Xing et al., 2014; Akkermann et al., 2016). However, the regeneration capacity and overall degree of successful remyelination is limited due to multiple inhibitory factors and immune reactions (Charles et al., 2002; Mi et al., 2005; Kuhlmann et al., 2008; Kotter et al., 2011; Kremer et al., 2011; de Castro et al., 2013; Gruchot et al., 2019). Mesenchymal stem cells are known to have strong pro-regenerative, anti-inflammatory properties (Uccelli et al., 2008; Maffioli et al., 2017; Spitzhorn et al., 2017; Teixeira et al., 2017; Baberg et al., 2019; Jungbluth et al., 2019; Timaner et al., 2020). Furthermore, MSC-secreted factors were shown to promote OPC maturation as well as the generation of oligodendroglia from adult NSCs (Rivera et al., 2006; Jadasz et al., 2013; Jadasz et al., 2018), thus providing opportunities to develop novel pro-regenerative treatment approaches aiming at myelin repair. However, the identity of MSC-secreted factors and underlying mechanisms fostering an oligodendroglial differentiation have not been identified so far (Rivera et al., 2008). Hence, the identification of secreted factors could provide important information on how myelin repair could be promoted. Furthermore, the functional degree of MSC-CM depending lineage instruction *in vivo* remained to be established.

In the present thesis, MSC-CM stimulated aNSCs were transplanted into different rodent CNS regions and survival time, integration, differentiation capacity and myelination ability were analyzed. The current results provide evidence that MSC-secreted factors can indeed promote oligodendrogenesis of transplanted aNSCs including the generation of myelinated segments *in vivo*. Furthermore, a comparative secretome and proteome analysis was performed in order to identify secreted proteins enriched in the MSC-secretome by mass spectrometry. Several secreted proteins were detected some of which have previously been shown to act on oligodendroglial survival and differentiation. The role of TIMP-1 in the MSC secretome was then tested in culture and *in vivo* confirming a strong impact on NSCs oligodendroglial differentiation capacity. Furthermore, secreted factors derived from

human iPSC-derived MSCs were also tested for their ability to induce a pro-oligodendroglial effect in aNSCs.

5.1 MSC-secreted factors promote oligodendroglial cell differentiation of aNSCs after transplantation into the rodent brain

Transfected aNSCs used for transplantation experiments were tested in culture reflecting a similar differentiation capacity as non-transfected cells (Jadasz et al., 2018). Since the UbC-StarTrack plasmids applied for these experiments were originally used for tracking cell lines by *in vivo*, *in vitro*, and *ex vivo* strategies, a general effect on the differentiation capacity or survival rate of transfected cells with this plasmids can be excluded (Figueres-Oñate et al., 2016). The hyperactive transposase of the PiggyBac system (hyPBase) achieves a heritable and stable labeling of cell progenies showed by the identification of fluorescent cells five month after *in utero* electroporation (Figueres-Oñate et al., 2016) which makes this system suitable for long-term cell tracking. However, in this study only low cell numbers were detectable at 7 days post transplantation into the GM and WM of the mouse brain. It should be noted that the survival rate did not depend on GM or WM localization reflecting previous studies (Vigano et al., 2013; Beyer et al., 2020). Compared to the present results, mouse aNSCs transplanted into the adult mouse brain survived a longer time period (Lois and Alvarez-Buylla, 1994; Herrera et al., 1999; Seidenfaden et al., 2006; Beyer et al., 2020). The low survival rate observed in the present study could be due to a strong host immune reaction since rat aNSCs were transplanted into the mouse brain. Nevertheless, other studies revealed a longer survival of interspecies transplanted cells, however, all animals obtained an immune suppressing treatment before and during the whole time of the experiments (Fricker et al., 1999; Karimi-Abdolrezaee et al., 2006; McGinley et al., 2017). Thus, for future transplantation experiments, an immunosuppressive treatment would be an option to counteract the cell number reduction.

After transplantation, enhanced oligodendroglial differentiation was detected after pre-stimulation with MSC-CM. A similar differentiation behavior was observed after *ex vivo* application of hippocampal aNSCs on cerebellar slices using stimulation with human MSC-CM (Jadasz et al., 2018) showing that even a reduced MSC-CM stimulation (50 % slice medium / 50 % MSC-CM) is sufficient to foster aNSCs towards a pro-oligodendroglial fate. These data suggest that MSC-secreted factors

are that prominent that low-level stimulation with MSC-CM is sufficient to initiate the process of oligodendrogenesis regardless of permanent stimulation.

Fate-mapping studies of OPCs revealed a region-dependent impact on OPC differentiation showing that most of the OPCs located in WM differentiate into mature myelinating oligodendrocytes while in the GM most of the cells remained NG2-positive OPCs (Dimou et al., 2008; Rivers et al., 2008). Furthermore, OPCs derived from WM are able to differentiate efficiently into mature myelinating oligodendrocytes in the WM but less in the GM indicating that the GM is a non-supportive environment regarding myelination (Vigano et al., 2013). While this study revealed no impact of the WM or GM on MSC-CM pre-treated aNSCs, regional differences were detectable after transplantation of p57kip2 suppressed aNSCs showing an increased differentiation toward myelinating oligodendrocytes after localization in WM compared to GM (Beyer et al., 2020). The p57kip2 gene encodes an intrinsic regulator of glial fate acquisition acting as a negative regulator of the oligodendroglial maturation (Jadasz et al., 2012; Beyer et al., 2020). It can therefore be speculated that MSC-derived pro-oligodendroglial factors dominate endogenous signals of the GM or WM.

It was previously shown that a 3-day pre-stimulation period is necessary to achieve an optimal pro-oligodendroglial impact of MSC-CM on aNSCs in culture (Jadasz et al., 2018). The present results demonstrated that the pro-oligodendroglial impact of MSC-secreted factors was already significant after a 1-day pre-treatment in culture when primed cells were implanted into the CNS (Figure 4.4; 4.5). Pro-oligodendroglial transcriptional signals are activated early after stimulation such as the oligodendroglial marker NG2, ceramide galactosyltransferase (CGT), Olig2, CNPase as well as MBP whereas astrocytic markers Id2 and Id4 are downregulated after one day of MSC-CM treatment in culture (Rivera et al., 2006; Jadasz et al., 2018) which seems to be stable in aNSCs after transplantation. Id2 and Id4 form complexes with Olig2 which blocks its ability to act as a transcription factor and thereby inhibiting the differentiation into oligodendrocytes (Samanta and Kessler, 2004), thus downregulating or blocking Id2 or Id4 would result in an increased oligodendroglial differentiation. That early induction of pro-oligodendroglial differentiation by MSC-CM stimulation thus robustly primes aNSCs differentiating into mature oligodendrocytes even after environmental changes which could depend on these early molecular alterations.

To further investigate whether cell survival or differentiation capacity are species-dependent, MSC-CM pre-stimulated rat aNSCs were transplanted into the intact rat brain revealing a low survival rate (see Figure 4.8). However, in comparison to transplantation into the intact mouse brain, rat aNSCs showed a better survival rate in the rat brain. It was recently demonstrated that mouse aNSCs transplanted into the intact mouse brain without immune-suppressive treatment survived up to 42 days after transplantation (Beyer et al., 2020). These data indicate that the survival of transplanted aNSCs seems to be species-dependent with mouse cells being transplanted into the mouse brain. While the results reflect the survival of transplanted aNSCs in a species-dependent manner, their differentiation capacity was not altered. Differentiation analysis of transplanted MSC-CM stimulated rat aNSCs into the rat brain revealed a pro-oligodendroglial differentiation at an expanse of astrocytes as shown after mouse transplantation. Since only few animals were used, the experiment will have to be repeated including higher animal numbers in order to confirm the observed species-dependent effects.

5.2 Spinal cord environment promotes the survival of transplanted aNSCs differentiating into mature myelinating oligodendrocytes

Since stimulated aNSCs survived only for a short period after transplantation into the rodent brain, MSC-CM pre-stimulated aNSCs were transplanted into the intact rat spinal cord to analyze whether aNSCs survive for a longer time in another CNS environment. Previous studies demonstrated a long-term survival of aNSCs after transplantation into the spinal cord. For example, rat fetal neural progenitor cells (NPCs) were detectable in the injured rat spinal cord expressing neuronal, astroglial and oligodendroglial markers five weeks post transplantation (Ogawa et al., 2002). Furthermore, NPCs were detectable up to 12 weeks survival time differentiating into mature oligodendrocytes (Karimi-Abdolrezaee et al., 2006; Parr et al., 2007; Parr et al., 2008). However, transplantation of neural stem cells into the intact spinal cord was performed only in few studies. Interestingly, human NSCs were detectable at 6 months after transplantation into the intact spinal cord (Yan et al., 2007) indicating that long survival times can indeed be achieved in both intact and injured spinal cord environment. In the present study, transplanted aNSCs survived at least for 28 days post grafting independent of α-MEM or MSC-CM pre-treatment (see Figure 4.9). However, the survival rate was diminished over time which could be due to an

immune response. Interestingly, co-transplantation of human umbilical cord blood (hUCB) derived MSCs and human NSCs (hNSCs) resulted in an improved cell survival rate which could be due to a more permissive microenvironment compared to single cell population transplantation (hUCB-MSCs or hNSCs alone) (Sun et al., 2019). These findings therefore suggest a future application of the pro-oligodendroglial capacity of MSC-CM to pre-stimulate NSCs and an additional co-transplantation of MSCs as an environmental modulating treatment. This could presumably result in an increased survival rate of NSCs, a better integration after transplantation and furthermore an effective treatment option.

In the present study, the spinal cord appeared to provide a better survival environment compared to the rodent brain which is probably due to a spinal cord specific immune response and environment compared to the brain (Xuan et al., 2019; Plemel et al., 2020). Microglia of the brain are different in development, cellular phenotype and biological function compared to spinal cord microglia (Xuan et al., 2019). In general, adult microglia of the spinal cord revealed differences in gene and receptor expression compared to brain microglia (de Haas et al., 2008; Olson, 2010; Wang et al., 2012; Li et al., 2016; Gosselin et al., 2017; Masuda et al., 2019) including a higher expression level of anti-inflammatory cluster differentiation (CD) 172a, CD200R and triggering receptor expressed on myeloid cells 2 (TREM2) (Hoek et al., 2000; van Beek et al., 2005; Takahashi et al., 2007b; Olson, 2010; Li et al., 2016) indicating that the naïve spinal cord could be anti-inflammatory. Furthermore, differences between brain and spinal cord microglia were detected during development. Here, the amount of microglia entering the spinal cord is much lower compared to the brain (Monier et al., 2007; Cifra et al., 2012), thus, the number of microglia could also be lower in the adult spinal cord which might explain the better survival rate of transplanted aNSCs. To proof this hypothesis, comparison of microglia invasion into the brain and spinal cord after transplantation of MSC-CM pre-stimulated aNSCs should be conducted in future.

In contrast, the immune response in the spinal cord seems to be stronger compared to the brain after injury (Schnell et al., 1999; Batchelor et al., 2008; Li et al., 2016; Liu et al., 2017; Xuan et al., 2019) including a stronger macrophage, microglia, and astrocyte activation and larger accumulation of neutrophils, T cells, and B cells compared to traumatic brain injury (TBI) independent of the time point after injury (Schnell et al., 1999; Batchelor et al., 2008; Zhang and Gensel, 2014). After spinal

cord injury (SCI), a widespread microglia activation and macrophage accumulation at the injury site was observed while, in contrast, TBI caused focal microglia activation and less accumulation of activated macrophages (Batchelor et al., 2008). Furthermore, CNS inflammation is linked to an enhanced blood-brain barrier (BBB) permeability (Scott et al., 2004). Due the extensive distribution of inflammatory cells after SCI compared to TBI it is conceivable that permeability differences between the BBB and blood–spinal cord barrier (BSCB) contribute to the more widespread inflammatory response after SCI (Zhang and Gensel, 2014). Differences between spinal cord and brain immune responses could possibly explain the differences in the survival rate shown in the present study. In order to assess the underlying reasons further studies investigating cell survival after transplantation into either intact brain or spinal cord should be performed in parallel addressing the different immune responses.

Interestingly, transplantation into the grey and white matter of the spinal cord revealed that aNSCs are preferentially localized in white matter whereas in the mouse brain no difference was detected. This preferred white matter localization is in agreement with previous observations demonstrating that embryonic hippocampal progenitor cells are preferentially located in the white matter after transplantation into the intact spinal cord (Enomoto et al., 2003). Furthermore, immortalized human NSC line transduced with a retroviral vector encoding Olig2 were observed to migrate from the injection site and to occupy preferentially white matter areas (Hwang et al., 2009). In this regard it is worth mentioning that OPCs were shown to migrate towards the white matter and were absent in the grey matter in the injured spinal cord (Baron-Van Evercooren et al., 1996).

Independent of the implanted spinal cord region, grafted aNSCs preferentially differentiated into mature oligodendrocytes at an expense of astrocytic cells (see Figure 4.10; 4.11). Although more mature oligodendrocytes were detected, no difference in the number of Olig2-expressing cells was observed. Since a small proportion of these Olig2-positive cells could also exhibit an astrocytic cell fate (Marshall et al., 2005; Cai et al., 2007; Tatsumi et al., 2018), it must be concluded that Olig2 expression alone not clearly depict an oligodendroglia lineage fate but rather a mixed population of both astrocytes and oligodendrocytes. It was demonstrated that cell fate determination depends on the regulation of Olig2 shuttling between cytosol and nucleus. A cytoplasmic localization of Olig2 was found in

cultured astrocytes and in a subset of astrocytes of the subventricular zone (Setoguchi and Kondo, 2004) while a nuclear expression correlates with an oligodendroglial cell fate. Nuclear accumulation of Olig2 in NSCs blocks the CNTF-induced astrocytic differentiation and translocation of Olig2 is promoted by activated AKT (Setoguchi and Kondo, 2004). In the present thesis, no differences between a nuclear and cytoplasmic expression of Olig2 were considered for evaluations which might be the reason why no differences of Olig2-expressing cells were found.

Previous studies revealed that besides differentiation into oligodendrocytes and astrocytes, NSCs co-cultivated with MSCs can also differentiate into neuronal cell types expressing doublecortin (DCX) and microtubule associated protein 2ab (Map2ab), however at low rates (Rivera et al., 2006; Steffenhagen et al., 2012). In contrast to previous published studies showing neuronal differentiation of NSCs after transplantation (Fricker et al., 1999; Seidenfaden et al., 2006; Yan et al., 2007; Furmanski et al., 2009), NSCs used in the present study were fostered to differentiate into oligodendroglial cell types after incubation with MSC-CM or into the astroglial lineage primed through α-MEM. Since the number of cells expressing oligodendroglial markers and cells positive for astrocytic markers is close to 100 % after transplantation of MSC-CM or α-MEM stimulated NSCs, detection of neuronal cell types is not assumed which was also confirmed by immunohistochemical analysis of neuronal markers (see Figure 4.12). Hence, neuronal differentiation after transplantation is diminished which is not opposed by environmental cues. In the injured spinal cord, rodent and human NSCs transplanted differentiate into myelinating oligodendrocytes as well as into neuronal cells (Ogawa et al., 2002; Cummings et al., 2005; Abematsu et al., 2010; Lu et al., 2012; Zhu et al., 2018). Whether MSC-CM pre-stimulated NSCs rather differentiate into oligodendroglial cells than into neuronal cells also in an injured environment needs to be investigated in future experiments.

To investigate whether MBP-expressing cells differentiate into functional oligodendrocytes, immunohistochemical analysis was performed revealing that MBP-positive protrusions of MSC-CM pre-stimulated cells appeared parallel to neurofilament-positive axons (see Figure 4.13A-A'') and further co-localized with the paranodal marker Caspr1 (see Figure 4.13B-B''). These paranodal structures were also observed after transplantation of NSCs into the injured hippocampus or in an EAE model indicating the ability of transplanted cells to functionally remyelinate

(Samanta et al., 2015; Koutsoudaki et al., 2016). Immunoelectron microscopy further revealed that MSC-CM pre-stimulated cells form myelin sheaths enwrapping axons with myelinating processes ending in paranodal loops after transplantation into the spinal cord (see Figure 4.14). Also transplanted rodent or human NPCs form effective myelin sheaths in the injured spinal cord and thus actively remyelinate (Keirstead et al., 2005; Cloutier et al., 2006; Karimi-Abdolrezaee et al., 2006) confirming the observations in the present study. In addition, accumulation of fluorescent protein localized in transplanted cells was in close association with host axons (see Figure 4.14C) similar to previously published data (Karimi-Abdolrezaee et al., 2006) indicating that myelin sheaths found in the present study are also functional.

Since immunoelectron microscopy was only performed with MSC-CM pre-stimulates aNSC animals future studies should include a comparative immunoelectron microscopy analysis of α-MEM pre-treated aNSC transplanted animals to quantitatively investigate the differences in functional maturation and myelin formation. In addition, functional differentiation of MSC-CM pre-stimulated aNSC- should be tested using an injury or demyelination model. Furthermore, the impact on endogenous progenitor or stem cells by direct cell–cell contact or by secretion of trophic factors, thus promoting endogenous remyelination capacity and functional regeneration should be investigated.

The pro-oligodendroglial features of MSCs could provide an opportunity to promote functional remyelination in demyelinating diseases either by a possible impact of transplanted cells on endogenous remyelination capacities (hence resident OPCs and aNSCs) or by the application of identified pro-oligodendroglial factors emanating from them. The strong impact of MSCs and MSC-secreted factors was previously demonstrated in the experimental allergic encephalitis model, a model for an inflammatory demyelinating disease. In this model, intravenous injection of human MSC-CM and MSCs led to functional repair by promoting the development of oligodendrocytes and neurons. MSCs were able to migrate sites of injury and modulated the disease progression and host immune response (Bai et al., 2009; Kemp et al., 2010; Shen et al., 2011; Bai et al., 2012; Rice et al., 2013; Sarkar et al., 2017). Therefore, the pro-oligodendroglial potential of MSC-CM pre-stimulated aNSCs and their integration capacity in the injured or demyelinated spinal cord as for example in a rat EAE model (Swanborg, 2001) or in a model of demyelinating

disorder of the brain induced by the toxin cuprizone (Torkildsen et al., 2008) should be evaluated.

5.3 MSC secrete a cocktail of oligodendroglial regulatory proteins

The underlying mechanisms and identity of proteins secreted by MSCs with strong and persisting pro-oligodendroglial impact on aNSCs in cell culture and *in vivo* have so far been unknown. Although several secretome analyses of MSC-secreted components already exist (Maffioli et al., 2017; Spitzhorn et al., 2017; Teixeira et al., 2017; Baberg et al., 2019; Jungbluth et al., 2019; Timaner et al., 2020) none of them specifically addressed their pro-oligodendroglial properties. To identify such secreted pro-oligodendroglial proteins a combined secretome and proteome analysis via mass spectrometry (Grube et al., 2018) was performed.

Comparative secretome analysis revealed 691 secreted proteins of which 152 proteins were identified as *bona fide* secreted due to the significant enrichment in MSC-CM (Table 10.1). From 152 proteins, 85% were predicted to be classically secreted since these proteins were predicted to have a signal peptide, 7% exhibited a transmembrane domain and 1% were predicted to be unconventionally secreted proteins (Figure 4.16). Most of the *bona fide* secreted proteins were assigned to GO-terms associated with extracellular environment, extracellular matrix (ECM) organization and cell adhesion. According to literature, several proteins identified as *bona fide* secreted could have an impact on oligodendrocyte differentiation or myelination (Table 4.1), e.g., chordin which is known to promote oligodendrogenesis from SVZ NSCs (Jablonska et al., 2010; Jadasz et al., 2012). However, chordin neutralization in MSC-CM had no impact on aNSC lineage differentiation (Figure 4.17) indicating that chordin might play only a minor role in MSC promoted oligodendrogenesis. Although antibody concentration was high in comparison to other studies with similar setups (Burgess et al., 2006; Schira et al., 2015; Silva et al., 2019) the antibody concentration could be too low to completely block chordin. Furthermore, the stimulation period of 3 days could be too short, e.g., the effect of blocking Lama2 in a pericyte-conditioned medium applied to NSCs was detectable after a minimum of 7 days in culture (Silva et al., 2019). In future experiments, antibody neutralization could be further optimized by testing other antibodies, further concentrations and extended time points.

Another ECM-associated protein positively regulating oligodendrogenesis is fibronectin which was found to maintain the OPC pool via promoting OPC proliferation (Lourenco et al., 2016). While fibronectin controls proliferation, syntenin-1 regulates migration of OPCs via the interaction with NG2 (Chatterjee et al., 2008). Furthermore, the ECM proteins Lamb2, osteopontin and prosaposin are known to be important for myelination (Hiraiwa et al., 1999; Chun et al., 2003; Selvaraju et al., 2004; Relucio et al., 2012; Kim et al., 2014; Meyer et al., 2014). Lamb2 was identified to be enriched in the MSC secretome, which is similar to Lama2 which was shown to play a role during oligodendrogenesis revealed by a pro-oligodendroglial differentiation of aNSCs after a pericyte-dependent stimulation (Silva et al., 2019). Furthermore, it is known that laminins regulate CNS myelination by interacting with integrin receptors as well as the non-integrin ECM receptor dystroglycan (Colognato et al., 2007), thus Lamb2 identified in the MSC secretome could have an impact on differentiation as well. OPCs and NSCs proliferation is promoted by an intracellular cleaved part of dystroglycan which was also enriched in the MSC secretome (Leiton et al., 2015; Ahmed et al., 2019). The MSC secretome further included osteopontin known to stimulate MBP synthesis and myelin sheath formation *in vitro* (Selvaraju et al., 2004), the myelinotrophic protein prosaposin, also known as sulfated glycoprotein 1, which promotes expression of myelin constituents and protects myelinating glial cells (Hiraiwa et al., 1999; Meyer et al., 2014) as well as the growth arrest-specific protein 6 (Gas6) which protects oligodendrocytes from TNF-α induced apoptosis (Shankar et al., 2006) and from cuprizone-induced demyelination (Binder et al., 2008). Another promising candidate protein was TIMP-1 known to be secreted by astrocytes and being neuroprotective via the regulation of matrix metalloproteinases (MMPs) (Tan et al., 2003). Furthermore, TIMP-1 was shown to promote oligodendrocyte differentiation and to enhance CNS myelination (Moore et al., 2011; Jiang et al., 2016; Nicaise et al., 2019). This functionality was confirmed in the here presented antibody depletion experiments showing an increased astrocytic differentiation in culture and after transplantation indicating that MSC-CM induced oligodendrocyte differentiation is, among others, mediated by TIMP-1. Thereby, the present thesis revealed that TIMP-1 is not barely secreted by astrocytes (Jiang et al., 2016) but also by MSCs. Antibody depletion experiments used in this study revealed an easy and efficient way to neutralize proteins of interest to test their effect on target cells. Furthermore, the transplantation paradigm used in this study represents a

method to analyze the effect of a specific pre-treatment and subsequent impact of an *in vivo* CNS environment. To further confirm the present results, future analyses should include a knockdown of TIMP-1 in MSCs as well as rescue experiments using recombinant TIMP-1 protein after TIMP-1 neutralization or knockdown. In addition, TIMP-1 could be applied to animals (either directly into the brain or via intrathecal injection) to analyze the impact of TIMP-1 on endogenous cells in wildtype rodents as well as in demyelination models.

Beside proteins likely to have positive impact on the oligodendroglial differentiation also proteins negatively regulating oligodendrogenesis were identified. ECM proteins ACN, TNC, THBS1 and BMP-1, IGFBPs and growth factors such CTGF were identified known to have a negative impact on oligodendrogenesis. While THBS1 and BMP-1 inhibit or reduce oligodendrogenesis (See et al., 2004; Lu and Kipnis, 2010) ACN and TNC were found to inhibit myelination (Czopka et al., 2010; Keough et al., 2016). IGFBP2 and -7 negatively affect oligodendrocyte survival and differentiation via the regulation of Wnt/β-catenin signaling (Kühl et al., 2002; Li et al., 2018) while CTGF was shown to negatively regulate myelination (Ercan et al., 2017). Nevertheless, the impact of the identified proteins with known oligodendroglial regulation have to be further evaluated with regard to their function in MSC-CM mediated oligodendrogenesis. For this purpose possible proteins could be destroglycan 1, Lamb2, osteopontin and CTGF.

Apart from the as *bona fide* identified proteins with known oligodendroglial regulatory roles, several proteins with unknown function in oligodendroglial differentiation need to be investigated (see Table 10.1), e.g. vitamin K-dependent protein S (ProS1) which regulates the balance between quiescence and proliferation of NSCs (Zelentsova et al., 2017), which have to be precisely regulated for differentiation, hence for oligodendrogenesis. Furthermore, is has been shown that activation of Tyro3 through ProS1 known to regulate myelination in the CNS (Akkermann et al., 2017; Shafit-Zagardo et al., 2018) which makes ProS1 a suitable candidate to investigate its functionality in relation to MSC-CM. Since laminin proteins are known to regulate CNS myelination (Colognato et al., 2007), the different laminin proteins identified as *bone fide* secreted should be investigated in future to characterize their functional role in oligodendroglial cell fate. Furthermore, Wnt-signaling is known to promote oligodendrogenesis (Ortega et al., 2013; Azim et al., 2014a; Azim et al., 2014b), thus, the negative regulator Dickkopf 3 (Dkk3) (Fancy et al., 2009) identified

in the MSC-secretome could be a negative regulator of oligodendrogenesis which needs to be further investigated.

In summary, the broad spectrum of negative and positive regulators of oligodendrocyte development and differentiation found in the MSC secretome suggests a balanced interplay of these factors to achieve a successful cell fate to purchase and establish newly formed myelin sheaths. In order to prepare a biomedical translation of such findings, future experiments investigating candidate proteins with putative positive or negative impact on the oligodendroglial cell fate of NSCs have to be conducted to identify rate-limiting components as well as those which might have the highest potential for a safe application *in vivo*. In this regard, route of administration will also have to be considered. Since transplantation of factors or cells directly into the brain or lesion side is out of scope for humans due to the invasiveness an intravenous or intranasal application would be therefore of advantage. Studies have already shown that intravenously applied MSC-CM or MSCs reach the target and promote remyelination (Bai et al., 2009; Bai et al., 2012). Furthermore, intravenous administration of sulforaphane, an activator of the nuclear factor erythroid 2–related factor 2 (Nrf2) resulted in protection of piglet brains from neonatal hypoxic-ischemic injury (Wang et al., 2020) revealing that single factors can be applied intravenously having effects in the target region. Furthermore, intranasal administration of MSCs was shown to preserve myelination after perinatal brain damage (Oppliger et al., 2016). It was further demonstrated that intranasal administration of IL4 nanoparticles after intracerebral hemorrhage facilitates hematoma resolution (Xu et al., 2020), confirming the functionality of this delivery method. Thus, intravenous or intranasal administration of cells or factors seems to be a promising method to possible treat diseases in the future.

5.4 Human iPSCs derived MSCs secrete pro-oligodendroglial factors

As previously shown, positive effects on oligodendroglial differentiation of MSC-CM are not restricted to rodent species since fetal human MSC-CM has the same potential to promote oligodendroglial differentiation and maturation (Jadasz et al., 2018). Interestingly, human iPSC-derived MSCs have the similar pro-regenerative properties as human MSCs (Spitzhorn et al., 2018; Jungbluth et al., 2019; Spitzhorn et al., 2019) which was also confirmed in this study in regards to their pro-oligodendroglial potential. Therefore, MSCs derived from iPSCs, reflecting human

MSCs also in terms of their pro-oligodendroglial potential, offer the possibility to investigate secreted factors from human MSCs which can be further used as future treatment approach for demyelinating diseases. In order to continue along this line the effect of iMSC-CM on aNSCs regarding survival, differentiation and tissue integration, transplantation experiments into the intact spinal cord or brain should be analyzed in future experiments using cell culture as well as *in vivo* paradigms. To investigate the potential of iMSC-secreted factors on oligodendrogenesis of aNSC and the functionality of such cells *in vivo,* further analysis should be performed in a naïve as well as the injured or demyelinated CNS. Moreover, future analyses should confirm candidate proteins already identified in the rodent MSC secretome. In addition, a mass spectrometry based iMSC secretome analysis possibly identify human specific proteins with pro-oligodendroglial characteristics.

5.5 Conclusion

The present thesis provides evidence that MSC-secreted proteins efficiently promote oligodendroglial cell fate of aNSCs *in vivo* which is based on positive and negative regulators identified by mass spectrometry based secretome analysis. MSC-secreted factors effectively initiate a pro-oligodendroglial cell fate of aNSCs even after a short stimulation which overcomes endogenous signals of the GM or WM resulting in functional myelin sheath formation *in vivo*. The pro-oligodendroglial properties of MSCs could promote functional remyelination in demyelinating diseases mediated either by their impact on cells to be transplanted or by application of identified pro-oligodendroglial factors. Furthermore, the immunomodulatory properties of MSCs could promote survival and remyelination after demyelination in a co-transplantation paradigm using MSC-CM pre-treated NSCs and MSCs.

Mass spectrometry-based secretome analysis revealed that the MSC-CM-mediated effect likely depends on a cocktail of proteins, suggesting a balanced interplay of negative and positive factors to achieve a functional oligodendrogenesis. Major component promoting a pro-oligodendroglial differentiation is TIMP-1. In future, TIMP-1 could be used to either promote endogenous oligodendrogenesis *in vivo* after demyelination or to foster transplanted stem cells towards an oligodendroglial lineage differentiation. In addition, the present study demonstrated that MSCs derived from iPSCs reflect the same pro-oligodendroglial properties as human or rodent MSCs showing that the effect on oligodendrogenesis is species-independent, providing new translational treatment opportunities to promote functional remyelination in demyelinating diseases.

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7. List of Figures and Tables

7.1 List of Figures

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8. Abbreviations

µg	microgram
ACN	aggrecan
AKT	serine-threonine kinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ang	angiogenin
Ang-1	angiopoietin 1
ANOVA	analysis of variance
aNSCs	adult the neural stem cells
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor, brain-derived neurotrophic factor
BL	black
Blbp	brain lipid binding protein
BMP	bone morphogenic protein
BSA	bovine serum albumin
BSCB	blood–spinal cord barrier
C	degree Celsius
Caspr	contactin associated protein/ paranodin/neurexin
CCL	CC-chemokine ligand
CCNT2	miR-297c-5p via interaction with cyclin T2
CD	cluster differentiation, cluster differentiation
CDK2	cyclin-dependent kinase-2
CGT	ceramide galactosyltransferase
Chrd	chordin
Cldn11	claudin-11
cm	centimeter
c-myc	v-myc myelocytomatosis viral oncogene homolog
CNPase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNTF	cytokine ciliary neurotrophic factor
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
CysC	cystatin C

Da	dalton
Dag1	dystroglycan 1, dystroglycan
DAPI	4´,6-diamidin-2-phenylindol
DCX	doublecortin
ddH_2O	double-distilled water
Dkk3	Dickkopf 3
Dlx2	distal-less homeobox 2
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNase	Deoxyribonuclease
EAE	experimental allergic encephalitis
ECM	extracellular matrix
EGF	epidermal growth factor
EPO	erythropoietin
ER	endoplasmic reticulum
ERas	embryonic stem cell-expressed Ras
ESCs	embryonic stem cells
Ezh2	enhancer of zeste homolog 2
FCS	Fetal calf serum
FGF	fibroblast growth factor
Fn	fibronectin
FoxO1	forkhead box protein O1
FoxP3	forkhead box P3
g	gram
GABA	gamma-aminobutyric acid
Gal-1	galectin-1
GalC	galactocerebroside C
Gas6	growth arrest-specific protein 6
GDF	growth differentiation factor
GDN	glia-derived nexin
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLAST	glutamate aspartate transporter

GM	grey matter
GO	Gene Ontology
GPR17	G protein-coupled receptor 17
GSK3β	glycogen synthase kinase 3β
GSTT1	glutathione S-transferase theta 1
GSTπ	glutathione-S-transferase-π
Gsx2	GS homeobox 2
h	hour, human
HBSS	Hank's Balanced Salt Solution
HCl	hydrochloric acid
HERV-W	human endogenous retrovirus type W
HLA	human leukocyte antigen
HSCs	hematopoietic stem cells
HSV-1	herpes simplex-1
hUCB	human umbilical cord blood
ICC	immunocytochemistry
IDO	indoleamine 2,3-dioxygenase
IFN-gamma	interferon-gamma
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor 1
Igfbp7	like insulin-like growth factor-binding proteins 7
IgG	immunoglobulins G, immunoglobulin G
IL	interleukin
IPCs	intermediate progenitor cells
iPSCs	induced pluripotent stem cells
ISCT	International Society of Cell Therapy
kg	kilogram
Klf4	krueppel-like factor 4
Lamb2	laminin subunit beta-2
LIF	leukemia inhibitory factor
LINGO-1	leucine rich repeat and immunoglobin-like domain-containing protein 1
LRP2	low-density lipoprotein-related protein 2
LSM	Laser-Scanning-Mikroskop
M	molar

MA	mantle
MAG	myelin-associated glycoprotein
Map2ab	microtubule associated protein 2ab
Mash1	achaete-scute complex homolog-1
MBP	myelin basic protein
mg	milligram
min	minute
mm	milliliter
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
MRF	myelin gene regulatory factor
MS	multiple sclerosis
MSC	mesenchymal stem cell
MSC-CM	MSC-conditioned medium
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
MyT1	myelin transcription factor 1
MZ	marginal zone
n	number of experiments
NaCl	sodium chloride
NB	Neurobasal medium
NE	neuroepithelium
Nf1	neurfibromin 1
NG2	neural/glial antigen 2
NGS	normal goat serum
nIPCs	neurogenic intermediate progenitor cell
nm	nanometer
NO	nitric oxide
Nrf2	erythroid 2–related factor 2
O4	O-antigen 4
OCT	optimal cutting temperature
Oct4	octamer binding transcription factor 4
oIPCs	oligodendrocytic intermediate progenitor cell
Olig2	oligodendrocyte transcription factor 2

OPCs	oligodendroglial progenitor cells
OPN	osteopontin
p57kip2	cyclin-dependent kinase inhibitor
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
Pbx1	B-cell leukemia homeodomain 1
PDGF	including platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PEDF	pigment epithelium-derived factor
Pen Strep	penicillin / streptomycin
PFA	paraformaldehyde
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLP	proteolipid protein
PP	primary progressive
ppm	parts per million
Prox1	prospero-related homeobox 1 gene
ps	pre-stimulation
PSA-NCAM	polysialic acid neural cell adhesion molecule
Psap	Sulfated glycoprotein 1 / Prosaposin
pt	post transplantation
RGs	radial glial cells
RMS	rostral migratory stream
RR	relapsing-remitting
RT	room temperature
SCF	stem cell factor
SCI	spinal cord injury
SDF-1	stromal cell-derived factor 1
SEM	standard error of the mean
serpin E1	serpin family member 1
SGZ	subgranular zone
Shh	sonic hedgehog
Sirt1	sirtuin-1
Sox	sex determining region Y-box transcription factor
SP	secondary progressive

Sp1	specificity protein 1
Spp1	osteopontin
SVZ	subventricular zone
TAPs	transit-amplifying progenitors
TBI	traumatic brain injury
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TCF4	transcription factor 4
TFA	trifluoroacetic acid
TGF-β	transforming growth factor β
Thbs1	thrombospondin 1
TIMP-1	tissue inhibitor of metalloproteinase 1
TNC	tenascin C
TNFα	and tumor necrosis factor α
TPA	tissue-type plasminogen activator
TREM2	triggering receptor expressed on myeloid cells 2
UbC	Ubiquitin C
Utf1	undifferentiated embryonic cell transcription factor 1
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VZ	ventricular zone
WM	white matter
Wnt	wingless-related integration site
zfp	zinc finger protein
α-MEM	minimum essential medium alpha
µl	microliter

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10. Annex

10.1 Table of enriched factors found in the MSC secretome

Table 10.1: 152 proteins were found to be enriched in the MSC secretome compared to the corresponding proteome

Protein name	Gene name	classes	Protein IDs
72 kDa type IV collagenase	Mmp2	SP1	P33436
A disintegrin and metalloproteinase with thrombospondin motifs 1	Adamts1	TM1	Q68EJ2
Adipocyte enhancer-binding protein 1	Aebp1	SP	A0A0H2UHL3
Aggrecan core protein	Acan	SP	P07897
Agrin	Agrn	TM	D4A2F1
Amyloid beta A4 protein	App	SP	Q6P6Q5
Apolipoprotein E	Apoe	SP	A0A0G2K151
Attractin	Atrn	IN	Q99J86
Beta-2-microglobulin	B2m	SP	P07151
Biglycan	Bgn	SP	P47853
Calsequestrin-2	Casq2	SP	P51868
Calsyntenin-1	Clstn1	SP	Q6Q0N0
Carboxypeptidase E	Cpe	SP	P15087
Cathepsin L1	Ctsl	SP	P07154
Chordin	Chrd	SP	A0A140TA94
Coiled-coil domain-containing protein 80	Ccdc80	SP	Q6QD51
Collagen alpha-1(I) chain	Col1a1	SP	P02454
Collagen alpha-1(III) chain	Col3a1	SP	P13941

Collagen alpha-1(V) chain	Col5a1	SP	G3V763
Collagen alpha-1(XI) chain	Col11a1	TM	P20909
Collagen alpha-2(I) chain	Col1a2	TM	F1LS40
Complement C1q tumor necrosis factor-related protein 5	C1qtnf5	SP	Q5FVH0
Connective tissue growth factor	Ctgf	SP	Q9R1E9
C-type lectin domain family 11 member A	Clec11a	SP	O88201
Cystatin-C	Cst3	SP	P14841
Elastin	Eln	SP	Q99372
Extracellular matrix protein 1	Ecm1	SP	Q62894
Fibromodulin	Fmod	SP	G3V6E7
Fibronectin type III domain-containing protein 1	Fndc1	SP	A0A0G2K4G5
Fibronectin	Fn1	SP	A0A096P6L8
Fibulin-5	Fbln5	SP	Q9WVH8
Follistatin-related protein 1	Fstl1	TM	F8WG88
Follistatin-related protein 3	Fstl3	SP	Q99PW7
Galectin-3-binding protein	Lgals3bp	SP	O70513
Glia-derived nexin	Serpine2	SP	G3V7Z4
Glypican-1	Gpc1	SP	Q6P7Q2
Granulins	Grn	SP	G3V8V1
Growth arrest-specific protein 6	Gas6	SP	Q63772
Immunoglobulin superfamily member 10	Igsf10	SP	G3V7S1
Inhibin beta A chain	Inhba	SP	P18331

Insulin-like growth factor-binding protein 2	Igfbp2	SP	P12843
Insulin-like growth factor-binding protein 3	Igfbp3	SP	P15473
Insulin-like growth factor-binding protein 4	Igfbp4	SP	P21744
Integrin beta-like protein 1	Itgbl1	SP	Q5PQQ8
Lactadherin	Mfge8	SP	P70490
Laminin subunit beta-2	Lamb2	SP	P15800
Latent-transforming growth factor beta-binding protein 1	Ltbp1	SP	D3ZAA3
Latent-transforming growth factor beta-binding protein 2	Ltbp2	SP	A0A0G2KAU7
Lumican	Lum	SP	P51886
Lysyl oxidase homolog 2	Loxl2	SP	B5DF27
Mannan-binding lectin serine protease 1	Masp1	SP	A0A0H2UHA1
Metalloendopeptidase	Tll1	SP	D3Z8U5
Metalloendopeptidase	Bmp1	SP	F1M798
Metalloproteinase inhibitor 1	Timp1	SP	P30120
Metalloproteinase inhibitor 2	Timp2	SP	P30121
Meteorin-like protein	Metrnl	SP	A0A0G2K1P2
Nidogen-2	Nid2	SP	B5DFC9
Nucleobindin-1	Nucb1	SP	Q63083
Olfactomedin-like protein 3	Olfml3	SP	B0BNI5
Osteopontin	Spp1	SP	P08721
Out at first protein homolog	Oaf	SP	Q6AYE5
Peptidyl-glycine alpha-amidating monooxygenase	Pam	SP	P14925

Plasminogen activator inhibitor 1	Serpine1	SP	P20961
Platelet-derived growth factor C	Pdgfc	IN	A0A0G2JTE4
Platelet-derived growth factor receptor-like protein	Pdgfrl	SP	Q5RJP7
Procollagen C-endopeptidase enhancer 1	Pcolce	SP	O08628
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Plod1	SP	D3ZQ74
Proprotein convertase subtilisin/kexin type 5	Pcsk5	SP	P41413
Protein FAM198B	Fam198b	TM	Q6P7B4
Protein NOV homolog	Nov	SP	Q9QZQ5
Protein-lysine 6-oxidase	Lox	SP	P16636
Renin receptor	Atp6ap2	SP	Q6AXS4
Ribonuclease 4	Rnase4	SP	O55004
Serine protease HTRA1	Htra1	SP	Q9QZK5
Serine/threonine-protein kinase BRSK1	Brsk1	IN	B2DD29
Signal recognition particle receptor subunit beta	Srprb	SP	Q4FZX7
Slit homolog 3 protein	Slit3	SP	O88280
SPARC	Sparc	SP	P16975
Sulfated glycoprotein 1	Psap	SP	F7EPE0
Sulfhydryl oxidase 1	Qsox1	SP	Q6IUU3
Sushi repeat-containing protein SRPX	Srpx	SP	Q63769
Sushi repeat-containing protein SRPX2	Srpx2	SP	B5DF94
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	Svep1	IN	A0A0G2JUE3;
Syntenin-1	Sdcbp	IN	Q9JI92

Thrombospondin-4	Thbs4	SP	F1LMS5
Transcobalamin-2	Tcn2	SP	Q9R0D6
Transforming growth factor beta-2	Tgfb2	SP	G3V6B1
Versican core protein	Vcan	SP	Q9ERB4
Vitamin K-dependent protein S	Pros1	SP	P53813
von Willebrand factor A domain-containing protein 1	Vwa1	SP	Q642A6
Collagen type IV alpha 2 chain	Col4a2	SP	F1M6Q3
Collagen type VIII alpha 2 chain	Col8a2	SP	D4ADG9
Insulin-like growth factor binding protein 7, isoform CRA_b	Igfbp7	SP	F1M9B2
Laminin subunit alpha 4	Lama4	SP	F1LTF8
Collagen type IV alpha 1 chain	Col4a1	SP	F1MA59
Dickkopf WNT-signaling pathway inhibitor 3	Dkk3	SP	B1H219
Laminin subunit beta 1	Lamb1	SP	D3ZQN7
Olfactomedin-like 2B	Olfml2b	SP	D4A0J7
Lysyl oxidase-like 1	Loxl1	SP	Q5FWS5
Lysyl oxidase-like 3	Loxl3	SP	D3ZP82
Pappalysin 2	Pappa2	SP	A0A0G2JU68
Osteoglycin	Ogn	SP	D3ZVB7
Matrix metallopeptidase 19	Mmp19	SP	C0M4B0
Periostin	Postn	SP	D3ZAF5
Cartilage intermediate layer protein	Cilp	SP	D3ZGB8
Semaphorin 3D	Sema3d	TM	F1MAG8

Scavenger receptor cysteine-rich family member with 5 domains	Ssc5d	SP	D3ZPK4
Thrombospondin 2	Thbs2	SP	D4A2G6
Alpha 4 type V collagen	Col5a3	SP	Q9JI04
Collagen type VI alpha 2 chain	Col6a2	SP	F1LNH3
Retinoic acid receptor responder (Tazarotene induced)	Rarres1	SP	Q58NB7
Collagen type V alpha 2 chain	Col5a2	SP	F1LQ00
Tenascin N	Tnn	SP	D3ZK14
C1q and TNF-related 3	C1qtnf3	SP	B1WC91
Dystroglycan 1	Dag1	SP	F1M8K0
Collagen type IV alpha 5 chain	Col4a5	SP	F1LUN5
LRRGT00160	Podn	IN	Q6QI48
Collagen type VIII alpha 1 chain	Col8a1	SP	D4AC70
Collagen type XVIII alpha 1 chain	Col18a1	SP	F1LR02
Exostosin glycosyltransferase 1	Ext1	TM	G3V901
Fibronectin	Fn1	SP	F1LST1
Peroxidasin	Pxdn	SP	A0A0G2JWB6
Collagen type XVI alpha 1 chain	Col16a1	SP	F1LND0
Laminin subunit alpha 5	Lama5	SP	F1MAN8
Fibrillin 1	Fbn1	SP	G3V9M6
Sushi domain containing 5 (Predicted)	Susd5	SP	D3ZSC1
Tenascin C	Tnc	SP	A0A0G2K1L0
Uncharacterized protein	Hspg2	SP	F1LTJ5

Retinoic acid receptor responder (Tazarotene induced) 2	Rarres2	SP	Q5BK77
Beta-1,4-galactosyltransferase 1	B4galt1	TM	G3V722
Latent-transforming growth factor beta-binding protein 3	Ltbp3	SP	F1LRT0
Collagen alpha-1(XII) chain	Col12a1	SP	A0A0G2KAJ7
Thrombospondin 3	Thbs3	SP	A0A0G2JZH3
Uncharacterized protein	Ndnf	IN	D4A6W5
Transforming growth factor, beta-induced	Tgfbi	SP	D4A8G5
Serine (Or cysteine) proteinase inhibitor, clade B, member 7	Serpinb7	TM	G3V6B5
EGF-containing fibulin extracellular matrix protein 2	Efemp2	SP	A0A0G2K2R5
Collagen type VI alpha 1 chain	Col6a1	SP	D3ZUL3
Complement C1r	C1r	IN	D4A1T6
Signal peptide, CUB domain and EGF-like domain-containing 2	Scube2	SP	D4AC02
Exostoses (Multiple) 2 (Predicted)	Ext2	TM	E9PTT2
Nidogen-1	Nid1	SP	F1LM84
Collagen alpha-1(XV) chain-like	Col15a1	IN	F1LPD0
Hemicentin 1	Hmcn1	SP	F1M4Q3
ABI family member 3-binding protein	Abi3bp	IN	F1M9R4
Laminin subunit gamma 1	Lamc1	SP	F1MAA7
Microfibril-associated protein 4	Mfap4	SP	G3V6A9
Complement C1s subcomponent	C1s	SP	G3V7L3
Thrombospondin 1	Thbs1	IN	M0R979

Peptidylprolyl isomerase	Fkbp10	SP	Q5U2V1
Complement C2	C2;Cfb	SP	Q6MG73
Alpha-2 antiplasmin	Serpinf1	SP	Q80ZA3

[1]SP signal peptide; [2]TM transmembrane; [3]IN Intracellular

11. Declaration of Authorship

Hiermit versichere ich, Iria Samper Agrelo, an Eides Statt, dass ich die vorliegende Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“ an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist.

Düsseldorf, den _____ _____

Iria Samper Agrelo