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

MESENCHYMAL STEM CELL GRAFTS PROMOTE OLIGODENDROGLIAL DIFFERENTIATION AFTER SPINAL CORD INJURY

Inaugural – Dissertation
zur Erlangung
des Doktorgrades der Humanmedizin

der
Fakultät für Medizin
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vorgelegt von
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Meinen Eltern

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SUMMARY

Im Rahmen traumatischer Rückenmarkverletzungen kommt es regelmäßig zu schweren und permanenten neurologischen Funktionsstörungen bzw. -ausfällen. Der irreversible Verlust ortsständiger Rückenmarkzellen einschließlich Neuronen, Astrozyten und Oligodendrozyten sowie die damit einhergehenden ausgeprägten strukturellen Veränderungen repräsentieren das morphologische Korrelat für die schweren funktionellen Beeinträchtigungen. Da das Rückenmark die Hauptverbindung zwischen dem peripheren Nervensystem und den Steuerungszentren des Zentralnervensystems (ZNS) darstellt, führen Verletzungen in unterschiedlichem Ausmaß zu sensiblen, motorischen sowie autonomen neurologischen Ausfällen. Auch bei schwersten Schädigungen des Rückenmarks bleibt bei einem Großteil der Patienten ein Teil der aufsteigenden bzw. absteigenden Axone erhalten. Allerdings kommt es häufig zur Demyelinisierung der verbliebenen Axone, so dass diese nicht zu einer möglichen funktionellen Erholung beitragen können. Obwohl spontan oligodendroglialer Zellersatz stattfindet, ist das adulte Rückenmark nicht in der Lage, den im Rahmen einer traumatischen Schädigung aufgetretenen Zelluntergang suffizient auszugleichen. Aus diesem Grund wird intensiv nach adäquaten Zellersatzstrategien gesucht. Stammzellen stellen hierfür einen vielversprechenden Therapieansatz dar.

Kürzlich konnte gezeigt werden, dass mesenchymale Stammzellen (MSC) einen bislang unbekannten Faktor sezernieren, welcher unter Ko-Kulturbedingungen die oligodendrogliale Differenzierung von adulten neuralen Vorläuferzellen aus dem Hippocampus sowie der Subventrikulärzone fördert und gleichzeitig die astrogliale Differenzierung dieser neuralen Vorläuferzellen inhibiert. In der vorliegenden Studie wurde zunächst der Einfluss von konditioniertem Medium der MSC (MSC-CM) auf das Differenzierungsverhalten von adulten neuralen Vorläuferzellen aus verschiedenen anatomischen ZNS-Regionen (Subventrikulärzone, Hippocampus, Rückenmark) unter Zellkulturbedingungen untersucht. Der vorbeschriebene prooligodendrogliale Effekt in Anwesenheit von MSC-CM konnte

erstmals auch in neuralen Vorläuferzellen aus dem Rückenmark sowie der subventrikulären Zone nachgewiesen werden. Im direkten Vergleich der einzelnen ZNS-Regionen bezüglich des Ausmaßes der Oligodendrogliogenese ergaben sich keine signifikanten Unterschiede.

Im Anschluss daran wurden die Effekte von MSC-Transplantaten auf die endogene Oligodendrogliogenese unmittelbar nach einer akuten traumatischen Rückenmarkverletzung analysiert. Im Vergleich zu Kontrolltieren, welche lediglich eine Rückenmarkläsion ohne Transplantat erhalten haben, steigern MSC-Transplantate das Ausmaß oligodendroglialer Differenzierung im Bereich der Rückenmarkläsion. Die gesteigerte oligodendrogliale Differenzierung ging, wie auch in vitro gezeigt, auf Kosten der astroglialen Differenzierung, die im Vergleich zu Kontrolltieren signifikant reduziert war. Die Ergebnisse der in-vitro Versuche legen die Vermutung nahe, dass diesem prooligodendroglialen Effekt ein von den MSC sezernierter löslicher Faktor als ursächlicher Mechanismus zugrunde liegt. Die exakte Identifizierung dieses Faktors ist im Hinblick auf ein genaueres Verständnis der Interaktionen zwischen MSC und endogenen neuralen Rückenmarkstammzellen von großer Bedeutung. Darüber hinaus müssen weiterführende Studien zeigen, ob die auf diese Weise neu entstandenen Oligodendrozyten in der Lage sind, die von der initialen Verletzung ausgesparten Axone zu remyelinisieren und dadurch zu einer funktionellen Erholung beitragen.

1. INTRODUCTION

1.1. Spinal cord injury

Spinal cord injury (SCI) regularly leads to severe and permanent disability. In most cases it is caused by a trauma resulting in vertebral fracturing with dislocation of bone fragments and consecutive contusion of spinal cord tissue. As the spinal cord represents the exclusive nerve conduction path between higher order brain structures and the peripheral nervous system, these injuries result in severe impairment of motor, sensory and autonomic function below the lesion site. Depending on the level and severity of injury, functional recovery can be achieved[1].

In patients with partial preservation of ascending and descending axon pathways across the lesion (incomplete SCI) limited spontaneous functional recovery is observed[2]. However, in more severe cases sensory, motor and autonomous function below the spinal cord lesion level is completely abolished (complete SCI). Effective regenerative therapies are not yet established. Interestingly, post mortem studies in subjects with functionally complete SCI revealed that despite complete loss of function a rim of still intact axon pathways across the lesion site is regularly observed[1,3,4]. However, the majority of preserved axons are demyelinated, which prevents proper nerve conduction and functional recovery. Therefore, therapeutic strategies aiming for remyelination of preserved axons might be able to promote partial functional recovery.

1.1.1. Epidemiology

The incidence of traumatic SCI worldwide varies between 12.1 to 57.8 cases per 1 million inhabitants in Western Europe respectively 25.2 to 52.5 in North America[5]. Lower incidence rates have been found in developing countries (12.7 to 29.7 per million inhabitants)[6]. Prevalence rates have shown a wide range from 130 up to 1124 per 1 million[7]. Common causes of traumatic SCI are motor vehicle accidents (42,1%), falls (26,7%; primary cause in

patients older 60 years), violence (15,1%) and sports activities (7,6%)[8]. The most common site of injury is the cervical spinal cord (around 54%), followed by thoracic (24%), lumbar (19%) and sacral (1%) spinal cord lesions[5]. In traumatic SCI studies two age peaks between 15 to 29 years and around 65 and older are observed[5]. The average age of patients suffering a SCI is around 33 years with a clear preference for males (2:1 to 7:1)[9,10,11]. However, there is a strong trend towards older individuals suffering from SCI within the last decade.

1.1.2. Classification of spinal cord injury

According to the American Spinal Injury Association Impairment Scale (ASIA) spinal cord injuries can be classified into 5 categories (ASIA A – E). ASIA A (45%) represents a functionally complete SCI with total loss of sensory and motor function below the level of injury. Incomplete spinal cord injuries start with ASIA B, being sensory incomplete but motor complete, all the way up to ASIA E. Each step (C, D, E) is characterized by an increasing motor score below the level of injury. Over the last decades injury severity shifted towards incomplete injuries, which account nowadays for almost two thirds of all traumatic spinal cord injuries[7,12].

1.1.3. Signs and symptoms of spinal cord injury

The clinical manifestation of SCI depends in particular on the level of injury. Cervical spinal cord lesions result in varying degrees of paresis and sensory impairment in the upper and lower extremities (tetraparesis or tetraplegia), whereas thoracic and lumbar lesions affect the trunk and lower extremities (paraparesis, paraplegia). Lesions at sacral level frequently affect exclusively bowel and bladder function. The interruption of the autonomic nervous system, which can happen at any level almost always causes neurogenic bladder and bowel dysfunction. In particular, cervical and high thoracic lesions are accompanied by cardiovascular dysfunction, so called autonomic dysregulation or autonomic dysreflexia.

In terms of mortality, the level of injury and the age of the patient at the time of injury are the most important factors[7]. The higher the lesion level, the lower the survival rate[13]. Patients with tetraplegia as a result of a cervical SCI are reported to have an almost 7-times higher mortality rate compared to patients with paraplegia. In terms of functional recovery, the level of incompleteness determines the respective prognosis. Less than 10% of SCI patients classified as ASIA A recover gait and standing function, whereas this rate increases dramatically in ASIA B, C and D patients[14].

1.1.4. Current concepts of spinal cord injury treatment

Established treatment of acute traumatic SCI consists of conservative and surgical (spine stabilization, decompression) measures including continuous monitoring on the intensive care unit to prevent cardiovascular, pulmonal and gastrointestinal complications. To compensate for lost function, to regain as much neurological function and to prevent secondary complications, neurorehabilitation in dedicated SCI centers, which provide highly spezialized medical and nursing care, physical therapy, occupational therapy, speech therapy, needs to be initiated as soon as possible. In particular, control of bowel/bladder/sexual function, spasticity and neuropathic pain need to be addressed in all patients with SCI. Up to now there is not a single established therapeutic intervention outside neurorehabilitation, which alters the course of the disease in a positive fashion. Therefore, regenerative therapies are urgently needed to promote recovery of function and quality of life in spinal cord injured subjects[15].

1.1.5. Pathomechanisms of spinal cord injury

In general three phases of SCI response can be observed. Acute, secondary and chronic injury processes[16-20]. The initial force (primary injury mechanism) causes immediate damage to the neural tissue (neurons, glia, long distance axons) and the vasculature of the spinal cord at the lesion level[16,21]. Subsequently, secondary injury mechanisms such as apoptosis[22-24], vascular changes[21,25-29], inhibition of intracellular protein synthesis, ionic dearrangements[30,31], free radicals[32-34] and glutaminergic excitoxicity[35-37] come into play. Immune cells become activated or migrate into the lesion to orchestrate an inflammatory reaction. This immune response is considered to have both beneficial (e.g. phagocytosis of axon growth inhibiting myelin debris[38,39] or limitation of secondary tissue damage by modulation of the T-cell response[40]) as well as negative effects (e.g. release of free radicals, enzymes and proinflammatory cytokines[41-44]) on recovery and functional outcome after SCI. In order to develop new treatment strategies in the future, it will be necessary to gain a deeper understanding of those complex secondary injury mechanisms and their relationship following acute SCI[7]. In the subacute to chronic phase lasting from days to years after the initial trauma the neural tissue in the lesioned spinal cord degenerates and a cystic lesion cavity surrounded by a fibroglial scar develops[45]. Apoptosis of oligodendroglia with consecutive demyelination of spared axons contributes to the failure of the injured spinal cord to recover spontaneously[1].

1.1.5.1. Growth inhibitory components and factors affecting intrinsic regenerative capacity

The cystic lesion defect developing within weeks after SCI represents a major obstacle for structural restoration following SCI. Neural tissue at the SCI lesion site lost due to necrosis and apoptosis is not replaced appropriately leaving a fluid filled cavity behind[46]. Subsequently, the initial cystic defect can further increase by ongoing apoptosis and release of

hydrolytic lysosomal enzymes[45,47,48]. The cystic lesion defect represents a physical barrier, which cannot be crossed by regrowing axons. Cell-based regenerative strategies try to overcome this barrier by providing a growth conducive environment, which might allow axon regeneration and functional recovery[49].

Lesion defects within the spinal cord are typically sealed off by a scar. This barrier consists of cellular and extracellular components. On the cellular side, astroglia and fibroblasts represent the main components[50,51]. Extracellular components such as chondroitin sulfate proteoglycans (e.g. neurocan, phosphocan, vesican, NG2 and aggrecan) support the scar[4,49,52,53,54]. Besides its negative effects as an axon growth inhibitory factor the glial scar plays an import role in stabilizing injured spinal cord tissue[55]. The scar supports the repair of the blood-brain-barrier, helps to contain the inflammatory response[56], protects spared neurons and oligodendrocytes by the secretion of growth factors and cytokines[57,53]. Molecular obstacles of regeneration are myelin-based inhibitors such as Nogo, Myelin-Associated Glycoprotein (MAG), tenascin-R and Oligodendrocyte Myelin glycoprotein (OMgp), which are released by damaged oligodendrocytes at the lesion site[58,59].

Numerous molecular changes have been discovered, which contribute to the intrinsic failure of injured central nervous system (CNS) axons to spontaneously regenerate. Neurite growth and regeneration associated genes are not upregulated as they should be[60-63]. Neurotrophic factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3/-4/5 (NT-3/-4/5), which are essential to maintain neuronal phenotypes and sustain synaptic functions during development, can also prevent axotomy induced neuronal atrophy after SCI[64-68].

1.1.5.2. Demyelination

Demyelination is observed in the course of the initial trauma and as part of secondary structural changes[4,69]. During the acute phase of SCI, necrosis more than apoptosis is responsible for cell death (especially within the surrounding grey matter), myelin breakdown and the consecutive loss of function in the vicinity of the primary lesion (primary demyelination). In addition the accumulation of macrophages and microglia within the lesioned area during the acute and subacute injury phase may also have a detrimental effect on the survival of damaged cells at the lesion center[70]. Nevertheless the loss of spinal cord cells and neurological function subsists for weeks to month due to secondary injury mechanisms (secondary demyelination). Especially myelin-forming oligodendrocytes ruled out to be very capable for those secondary degeneration processes[20,23,71-74]. In a rat spinal cord contusion model, Totoui et al. demonstrated that SCI is accompanied by progressive demyelination[75]. Within days to weeks after SCI axonal degeneration and myelin breakdown can also be found in long white matter tracts remote from the initial lesion[76,77]. Most likely ongoing apoptosis plays a central role within this process of chronic demyelination after SCI[78]. Especially the long-term apoptotic death of myelin-forming oligodendrocytes in long fiber tracts undergoing Wallerian degeneration significantly contributes to demyelination of spared axonal pathways remote from the lesion epicenter contributing to permanent neurological dysfunction[79]. These findings have been observed both in experimental SCI in small animals such as rats and mice[23,80-84] and human SCI[19,74], suggesting there might be a therapeutic window to protect oligodendroglia and reduce demyelination after SCI in humans.

What is the ideal spinal cord injury model to study demyelination and consecutively remyelination strategies? A recent study demonstrates that contusion SCI substantially differs from transection SCI, resulting in a more widespread initial mechanical trauma, leading to apoptosis of oligodendrocytes at greater distances (several millimeters) rostral and caudal from the lesion epicenter with consecutive demyelination. Apparently the extent of the initial

mechanical injury is highly correlated with the extent of demyelination[85]. As opposed to highly standardized and uniform experimental SCI one has to keep in mind that injury mechanisms vary significantly in human SCI, which most likely results in a wide range of demyelination extent[2]. Post mortem studies of injured spinal cords in humans have shown that in clinically complete spinal cord injured patients a significant number of spared axons is frequently observed (so called anatomically "discomplete")[2,3,77,86]. The majority of these spared axons are typically demyelinated, which prohibits proper nerve conduction. Thus, despite axon sparing preserved pathways are unable to mediate functional recovery[85,87,88].

1.1.5.3. Remyelination

Remyelination is a spontaneous process that follows demyelination[69,75,85,89,90]. Reports on remyelination inside the damaged CNS date back to the 1960s[91,92]. Over the last decades oligodendroglial progenitor cells (OPC) have been identified as the source of CNS remyelination[93]. In terms of remyelination of spared axons it is essential that OPC differentiate into mature myelin-forming oligodendrocytes following their recruitment and migration from the subependymal layer of the spinal cord central canal towards the lesioned area. This process encompasses a couple of steps: First a contact with the demyelinated axon has to be established, followed by the expression of myelin genes and the generation of myelin membranes, and finally wrapping and compacting those membranes to ultimately form the myelin sheat[94]. Interestingly several experimental models of chemically-induced demyelination resulted in complete remyelination by endogenous progenitor cells[85,95-97]. However, there is only poor remyelination after SCI leaving permanently demyelinated axons behind[69,75,90].

A lack or deficiency in precursor cells, a failure of precursor cell recruitment and a failure of precursor cell differentiation and maturation have been identified as reasons for poor remyelination. In particular, insufficient terminal oligodendroglial differentiation appears as

the most relevant factor, which prevents the formation of myelin and consecutive restoration of nerve conduction[94]. These findings are based on the detection of oligodendroglial lineage cells that are apparently unable to fully differentiate in areas of demyelination[98,99]. As underlying mechanism for the insufficient oligodendroglial differentiaton several factors have been identified. Especially the expression of myelin-associated proteins after SCI inhibits terminal differentiation into fully myelinating oligodendroglia[100-102].

Taken together, the extent of demyelination and insufficient endogenous remyelination shown in experimental SCI provide a relevant target for regenerative therapeutic interventions.

1.2. Regenerative strategies after spinal cord injury

Strategies to promote structural regeneration following SCI include axonal repair, neuronal replacement and remyelination strategies[2,3,77,103,104]. The following chapter will address these different repair strategies in more detail.

1.2.1. Axonal repair

The breakdown of communication between the peripheral nervous system and the brain is mainly caused by the interruption of long ascending (sensory) and descending (motor) axonal pathways[105]. It has been shown that recovery after SCI is significantly correlated with the amount of surviving axons[106,107]. Therefore, strategies to promote axonal plasticity and regeneration represent the prime target for innovative therapeutic concepts[1,3,4]. Various neurotrophic factors have been shown to enhance spinal cord plasticity and axonal outgrowth so far (e.g. the upregulation of neurite growth and regeneration associated genes)[60-63,65-67,108-113]. Neutralization of molecules inhibiting axonal regeneration such as CSPG (chondroitin sulfate proteoglycans) and myelin-based inhibitors represents another strategy to elicit axon repair[114-124].

The injured adult mammalian spinal cord parenchyma is unable to replace lost tissue in a phenotypically appropriate fashion. Therefore, efficient cell/tissue replacement strategies at the lesion site are required to promote axon regeneration with consecutive functional repair after SCI. Both, cell and biomaterial based transplantation/implantation therapies are effective to promote axon regrowth/sprouting to a limited extent[1,49,52,125-129].

1.2.2. Strategies for remyelination

SCI results in loss of oligodendrocytes, demyelination of spared axons and severe functional impairment. Since the adult CNS provides only a very limited capacity for spontaneous remyelination (especially since the terminal differentiation of endogenous progenitor cells

into mature myelin-forming oligodendrocytes is often not sufficient), cell transplantation strategies are an attractive approach for myelin repair. Very encouraging results originate from two major therapeutic approaches: 1) transplantation of cells with the potential to myelinate spared axons 2) stimulation of endogenous spinal cord progenitor cells to differentiate into mature myelin-forming oligodendrocytes.

1.2.2.1. Transplantation of myelin-forming cells

Several cell types have been transplanted to the lesioned spinal cord to extrinsically replace myelin-forming cells. Neural stem cells can differentiate into oligodendrocytes, which are capable to remyelinate axons. However, in most instances spontaneous differentiation into mature oligodendroglia is sparse[130,131]. A variety of strategies has been employed to enhance oligodendroglial differentiation of grafted neural stem/progenitor cells such as the administration of growth factors[132], the manipulation of gene expression or co-grafting of other cell types[108,133-138].

Neural stem cell transplantation approaches either alone or in combination with growth factors induced limited remyelination and functional recovery in small animal models of SCI[128].

Outside of the CNS Schwann cells represent the only other cell type which forms myelin. Interestingly the formation of myelin by Schwann cells is not limited to the peripheral nervous system only, but can also be observed in the CNS following invasion or grafting of Schwann cells to the injured spinal cord. Numerous studies revealed their potential to form functionally relevant myelin-sheets with improvement of saltatory nerve conduction velocity after transplantation to different CNS areas including the spinal cord[133,139,140]. Similar results have been published with olfactory ensheating cells[141-144], which can be considered as a hybrid cell between peripheral nervous system (Schwann cell) and CNS (astrocytes) glia.

1.2.2.2. Stimulation of endogenous progenitor cells for remyelination

Various studies have focused on maximizing the regenerative potential of endogenous neural progenitor cells (NPC) in the spinal cord by stimulating their recruitment, proliferation, migration and differentiation to intrinsically restore lost tissue following SCI[1,145].

The creation of a microenvironment conducive to regeneration by endogenous spinal cord stem cells with consecutive remyelination of spared axons plays a central role in this remyelination strategy. This goal could be achieved by local changes at the lesion site, probably mediated indirectly by the production of growth factors, cytokines, neurotrophic factors, favorable substances for axonal growth, effects on vasculature and other promoters of neurological recovery[1,94,146].

Recently, mesenchymal stem cells (MSC) have been shown to secrete soluble factors, which strongly promote oligodendroglial fate decision in adult NPC derived from the hippocampus (HC) or the subventricular zone (SVZ) in vitro. The shift of differentiation towards the oligodendroglial lineage has been demonstrated by a highly significant increase of the oligodendroglial markers MBP (myelin basic protein) and GalC (galactocerebroside) at the expense of astrogenesis, which was confirmed by a reduction of newborn GFAP-expressing astrocytes (glial fibrillary acidic protein). Subsequent experiments showed that enhanced oligodendroglial differentiation can be recapitulated on postnatal hippocampal slices cultures, which were incubated with MSC and NPC[138,147,148]. The exact factor to exert remyelination has yet to be identified[149].

Therefore, the transplantation of MSC or administration of MSC-CM (mesenchymal stem cell – conditioned medium) might also have the capacity to promote oligodendroglial differentiation in vivo. However MSC failed to enhance oligodendroglial differentiation of co-grafted exogenous NPC after transplantation to the acutely injured rat spinal cord[137,138].

The aim of the present study is to investigate whether pure MSC grafts will promote oligodendroglial differentiation in *endogenous* glial progenitor cells after spinal cord injury.

2. AIM OF THE THESIS

The aim of the thesis project is to investigate whether MSC grafted into the injured rat spinal cord will promote oligodendroglial differentiation in endogenous glial progenitor cells as prerequesite for enhanced remyelination.

3. MATERIAL & METHODS

3.1.Chemicals & Materials

3.1.1. Cell culture

Chemicals	Source
Alpha-MEM	Gibco BRL, Germany
Accutase	PAA Laboratories, Linz, Austria
Acepromazine	Sanofi-Ceva, Düsseldorf, Germany
B27 supplement	Gibco BRL, Germany
Boric acid	Sigma-Aldrich, Taufkirchen, Germany
Bromodesoxyuridine (BrdU)	Sigma-Aldrich, Taufkirchen, Germany
Dispase II	Boehringer, Germany
DMEM/F12	PAN Biotech GmbH, Aidenbach, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen, Germany
DNase I	Worthington Biochemicals, England
Dulbecco´s phosphate buffered saline (DPBS)	PAA Laboratories, Linz, Austria
Epidermal growth factor (EGF)	R&D Systems, Germany
Fetal bovine serum (FBS)	PAN Biotech GmbH, Aidenbach, Germany
Fibroblast growth factor (FGF)	R&D Systems, Germany
G418 (geneticin)	Invitrogen / Gibco, Karlsruhe, Germany
Glucose	Merck, Germany
Hank´s Balanced Salt Solution (HBSS)	PAA Laboratories, Linz, Austria
Heparin	Sigma-Aldrich, Taufkirchen, Germany
Ketamine	WDT, Garbsen, Germany
Laminin	Sigma-Aldrich, Taufkirchen, Germany
L-glutamine	PAN Biotech GmbH, Aidenbach, Germany
NaH_2PO_4	Sigma-Aldrich, Taufkirchen, Germany
Na_2HPO_4	Sigma-Aldrich, Taufkirchen, Germany
Neurobasal medium (NB)	Gibco BRL, Germany
Papain	Worthington Biochemicals, England
Penicillin/streptomycin	PAN Biotech GmbH, Aidenbach, Germany
Polybrene (hexadimethrine bromide)	Sigma-Aldrich, Taufkirchen, Germany
Poly-L-ornithine	Sigma-Aldrich, Taufkirchen, Germany
Sodium hydroxide (NaOH)	Sigma-Aldrich, Taufkirchen, Germany
Trypan blue	Sigma-Aldrich, Taufkirchen, Germany
Trypsin	Gibco BRL, Germany

3.1.2. Immunodetection

Chemicals	Source
3,3´-Diaminobenzidine (DAB)	Vector Laboratories, Burlingame, USA
4,6´-Diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Taufkirchen, Germany
Bovine serum albumin (BSA)	Biomol, Germany
DAB Peroxidase Substrate Kit	Biozol, Eching, Germany
Donkey serum	PAN Biotech GmbH, Aidenbach, Germany

Gelatine from cold water fish skin	Sigma-Aldrich, Taufkirchen, Germany
Prolong Antifade	Invitrogen GmbH, Germany
Triton X-100	Sigma-Aldrich, Taufkirchen, Germany
Vectastain Elite ABC kit	Vector Laboratories, Burlingame, USA
Xylazine	WDT, Garbsen, Germany

3.1.3. Other Chemicals + Kits

Chemicals	**Source**
Ethanol	Baker, Unterschleißheim, Germany
2-Mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
2-Methylbutane	Fluka
Paraformaldehyde	Sigma-Aldrich, Taufkirchen, Germany

3.1.4. Antibodies

Primary antibody	**Dilution**	**Source**
ms α APC (adenomatous polyposis coli)	1:500	Calbiochemi, Darmstadt, Germany
rt α BrdU (Bromodesoxyuridine)	1:500	AbDSerotec, UK
rb α GFAP (glial fibrillary acidic protein)	1:1000	Dako, A/S, Glostrup, DK
gt α GFP (green fluorescent protein)	1:500	Rockland, Gilbertsville, USA
ms α Map2ab (microtuble associated protein 2ab)	1:250	Sigma-Aldrich, Taufkirchen, Germany
ms α MBP (myelin basic protein)	1:750	HiSS Diagnostics GmbH, Germany

Secondary antibody	**Dilution**	**Source**
dk α ms IgG Alexa Fluor 488	1:1000	Molecular Probes, Germany
dk α gt IgG Alexa Fluor 488	1:1000	Molecular Probes, Germany
dk α rb IgG Alexa Fluor 488	1:1000	Molecular Probes, Germany
dk α ms-RHOX (rhodamine X)	1:500	Dianova, Hamburg, Germany
dk α rb-RHOX	1:500	Dianova, Hamburg, Germany
dk α rt-RHOX	1:500	Dianova, Hamburg, Germany
dk α rb-CY5	1:500	Dianova, Hamburg, Germany
dk α rt-Biotin	1:500	Dianova, Hamburg, Germany

3.1.5. Buffer and solutions

Reagent	Application
Borate buffer (0,1M; pH 8.5)	- 3.08g boric acid - 450ml H_2O - 5M NaOH to pH 8.5 - fill up to 500ml
Cryoprotect solution (CPS)	- 250ml Glycerine - 500ml 0,1M PO_4 buffer - 250ml ethylene glycol
Donkey serum blocking buffer	- 960µl TBS - 30µl donkey serum - 10µl Triton X-100
Fish skin gelatine buffer (FSGB)	- 1000ml TBS - 10g bovine serum albumin (BSA) - 2ml Fish skin gelatine - optional: 1ml Triton X-100
Phosphate buffer (PO_4 0,2M)	- 6.35g NaH_2PO_4 x H_2O - 41.35g Na_2HPO_4 x 7 H_2O - fill up with ddH_2O to 1L
Paraformaldehyde 4% (PFA)	- 40g paraformaldehyde - add 500ml H_2O - heat to 70°C - dissolve while stirring - add 1ml 10M NaOH - filter solution and add 500ml 0.2M PO_4
Phosphate buffered saline 0,1M (PBS)	- 500ml 0,2M phosphate buffer - 500ml dH_2O - 9g sodiumchloride
PPD (100ml)	- 0.01% Papain - 0.1% Dispase II - 0.01% DNase - 149mg $MgSO_4$ x 7 H_2O - in HBSS w/o Ca^{2+}/Mg^{2+}
Sucrose 30%	- 300g Sucrose - 400ml 0,2M PO_4 - 400ml ddH_2O
20 x SCC	- NaCl 175.3g - 88.2g Trisodium citrate x 2 H_2O - fill up with dH_2O to 1L
10 x TBS (Tris buffered saline)	- Trizma Base 30g - KCl 2g - NaCl 80g - fill up to 1L with dH_2O - adjust pH 7.4 with 1M HCl

3.1.6. Consumables

Materials	Source
Coverslips and slides	VWR International
Cell culture articles	Peske, Aindlingen-Arnhofen, Germany Corning Costar, Germany
Cell culture media	PAN Biotech GmbH, Aidenbach, Germany Invitrogen / Gibco, Karlsruhe, Germany
Growth factors	R&D Systems, Germany
Gelfoam (Gelita tampon)	Braun, Germany

3.1.7. Software

Program	Software producer
Adobe Illustrator CS3	Adobe, San Jose, USA
Adobe Photoshop CS3	Adobe, San Jose, USA
EndNote X3	Thomson Reuters, USA
Microsoft Office 2008 for Mac OS X (12.2.5)	Microsoft Corporation, USA
Prism 4 (Version 4.0a for Macintosh)	GraphPad Software, San Diego, USA
Spot 3.5.9 for Mac OS	Diagnostic Instruments, Sterling Heights, USA
Leica confocal software	Leica, Wetzlar, Germany

3.1.8. Equipment and instruments

Apparatus	Source
Hera Cell incubator	Heraeus, Germany
Hera Safe cell culture hood	Heraeus, Germany
Stereotactic unit	Kopf Instruments, Tujunga, USA
Tungsten wire knife	Kopf Instruments, Tujunga, USA
Picospritzer Π	General Valve, Fairfield, USA
Inverse fluorescence microscope for cell culture	Olympus, Germany
Leica confacal fluorescence microscope (TCS-NT)	Leica, Wetzlar, Germany
Leica fluorescent microscope equipped with a Spot CCD camera model 2.2.1	Leica, Wetzlar, Germany Diagnostic Instruments, Sterling Heights, USA
FACSAria	Becton Dickinson, USA
Refrigerated table centrifuge	Eppendorf, Hamburg, Germany
Table centrifuge mini spin plus	Eppendorf, Hamburg, Germany

3.2. Methods

3.2.1. Animal subjects

For all experiments, adult female Fischer 344 rats (Charles River Deutschland GmbH, Sulzfeld, Germany) weighing between 160-180g (3-4 months old) were used as donors for isolation of NPC, MSC and fibroblasts (FF) as well as for transplantation experiments. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. Animals had *ad libidium* access to food and water throughout the study. All efforts were made to minimize the number of animals and their suffering.

3.2.2. Preparation and cell culture

3.2.2.1. Preparation of neural progenitor cells (NPC)

For isolation of the different cell types, adult female Fischer-344 rats were deeply anesthetized using a mixture of ketamine (62.5 mg/kg), xylazine (3.175 mg/kg) and acepromazine (0.625 mg/kg) in 0.9% sterile saline solution and killed by decapitation. Brains and spinal cords were removed and put into Dulbecco´s phosphate buffered saline (DPBS) at 4°C. Overlying meninges and blood vessels were removed. The HC and the ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle (SVZ), were aseptically removed. The dissected tissue was transferred to fresh DPBS, washed once, afterwards transferred to Petri dishes and dissociated mechanically. The cell suspension was washed in DPBS to rinse of excess of blood and further digested in PPD solution containing 0.01% papain, 0.1% dispase II, 0.01% DNase I and 12.4 m$MgSO_4$, dissolved in Hank´s balanced salt solution (HBSS) for 30 min at 37°C. Thereafter the cell suspension was triturated every 10 min until the tissue was digested completely. The tissue was centrifuged at 120 x g for 5 min at 4°C and washed three times in Neurobasal (NB) medium supplemented with B27, 2 mM L-glutamine, 100 U/ml penicillin / 0.1 mg/l streptomycin. Cells were

resuspended in NB medium supplemented additionally with 2 µg/ml heparin, 20 ng/ml FGF-2 and 20 ng/ml EGF. Singe cells started to form spheres within one week of suspension culture. Cultures were maintained as neurospheres in uncoated culture flasks at 37°C in a humidified incubator with 5% CO_2. Half of the cell culture medium was changed twice a week, by centrifuging the medium containing the neurospheres at 120 x g for 5 min at 4°C and thereafter removing the supernatant and resuspending the cells in fresh growth medium[150,151]. Cell cultures were passaged in weekly intervals. Passaging of neurospheres was performed as follows: the medium containing the neurospheres was collected in a 15 ml centrifuge tube and centrifuged at 120 g x 5 min at 4°C. The cell-pellet was resuspended in 200 µl of Accutase™ and incubated at 37°C for 10 min. The neurospheres were resuspended in growth medium and triturated. Viable cells were counted by trypan blue exclusion assay in a Neubauer hemocytometer and a total number of 5 x 10^4 cells/ml was seeded in T75 culture flasks in fresh growth medium. To label cells, NPC were genetically modified to express the reporter gene GFP as described[46]. Briefly, NPC were plated in sub-confluent densities (10.000 cells/cm^2) on P-Ornithin/Laminin-coated cell culture flasks. The cells were incubated for 8 hours on two consecutive days with retrovirus containing supernatants (pCLE-GFP or pLXSNGFP) supplemented with 1 µg/ml Polybrene. Approximately 60% of all cells displayed GFP fluorescence 2 days following the transduction. To select for GFP-expressing cells that integrated the retroviral vector, G418 (500 µg/ml active concentration) was added to the growth medium for 6 weeks. This concentration has previously been determined to completely eliminate all untransfected NPC within 3 weeks. Successful incorporation of the transgene into all NPC was confirmed by an inverted fluorescence microscope (Olympus, Germany). Following G418 selection, cells were cultivated in the absence of G418.

3.2.2.2. Preparation of fibroblasts (FF)

Primary cultures of adult Fischer 344 fibroblasts were generated from skin biopsies and cultivated under standard culture conditions as previously described[130,152]. A small skin biopsy was taken after the fur over the abdominal region was shaved. The biopsy was put into 70% ethanol for a few seconds, washed in Hank´s balanced salt solution and fat tissue was removed. The biopsy was then cut into small pieces (1 x 1 mm) and transferred in culture wells containing Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin / 0.1 mg/l streptomycin, 3.5 mg/ml Glucose and 10% fetal bovine serum. The medium was changed twice a week. When cells reached 90% confluence, they were passaged by trypsinization (0.25% Trypsin). After counting an aliquot of the resulting single cell suspension in a Neubauer hemocytometer, 5 x 10^4 cells/ml were plated in fresh medium.

3.2.2.3. Preparation of mesenchymal stem cells (MSC)

MSC were isolated from bone marrow plugs harvested from femurs and tibias of Fischer 344 rats (2-4 months old). Plugs were mechanically dissociated in alpha minimum essential medium (αMEM) and recovered by centrifugation. Cell pellets were resuspended in αMEM containing 10% fetal bovine serum (αMEM-10% FBS) and seeded at a density of 1x10^6 cells/cm^2 in a humidified incubator at 37°C with 5% CO_2. After 3 days medium was changed and non-adherent cells were removed. Adherent cells were incubated in fresh αMEM-10% FBS until a confluent layer of cells was reached. These cells were trypsinized using 0.25% Trypsin and seeded in αMEM-10% FBS at 8.000 cells/cm^2. After 3-5 days of culture, the resulting monolayer of cells, hereafter named rat bone marrow-derived MSC, was trypsinized and frozen or cultured for further experiments. As demonstrated previously, this cell culture preparation is highly enriched with multipotent MSC containing virtually no hematopoietic contamination[147].

3.2.2.4. Preparation and use of conditioned media

For preparation of the MSC-CM and fibroblast-conditioned medium (FF-CM), each cell type was plated at 12.000 cells/cm^2 and incubated in αMEM-10% FBS. After 3 days, MSC-CM and FF-CM were collected and filtered using a 0.22 μm pore filter.

3.2.2.5. In vitro differentiation assays

NPC were plated on polyornithine (100 μg/ml) and laminin (5 μg/ml)-coated glass coverslips at a concentration of 5.000 cells/ml in α-MEM–10% FBS for 12-24 hours. Thereafter the medium was replaced with MSC-CM or FF-CM. NPC, which were kept in α-MEM–10% FBS medium served as controls. After 7 days cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (37°C, pH 7.4) and then processed for immunocytochemistry.

3.2.3. Immunocytochemistry

Fixed cells were washed in Tris-buffered saline (TBS) (0.15 M NaCl, 0.1 M Tris-HCl, pH=7.5), then blocked with a solution composed of TBS, 0.1% Triton X-100 (only for intracellular antigens), 1% bovine serum albumin (BSA) and 0.2% fishskin gelatine buffer (FSGB). The latter solution was used for the following incubation steps with antibodies. Primary antibodies were applied over night at 4°C. Fluorochrome-conjugated species-specific secondary antibodies were used for visualization.

The following primary antibodies and final concentrations have been used: rabbit anti-glial fibrillary acidic protein (GFAP) for astroglia (1:1000), mouse anti-myelin basic protein (MBP) (1:750; SMI-94) for oligodendroglia and mouse anti-microtubule associated protein 2ab (Map2ab) (1:250) for neurons. Goat anti-green fluorescent protein (GFP) (1:500) was used to detect GFP-positive NPC. Secondary antibodies: donkey anti-mouse, -rabbit or -goat conjugated with rhodamine X (RHOX) (1:500) or Alexa fluor 488 (1:1000). Nuclear staining was performed with 4',6'-diamidino-2-phenylindole-dihydrochloride-hydrate (DAPI) at 0.25 µg/µl. Coverslips were mounted on microscope slides using Prolong Antifade. Epifluorescence observations and photodocumentations were realized using a Leica fluorescent microscope (Leica, Wetzlar, Germany) equipped with a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Inc., Sterling Heights, USA).

For each culture condition randomly selected observation fields, containing five hundred to 1.000 GFP-positive cells, were photographed for cell fate analysis. The expression frequency of selected cell type markers was determined for every condition in three independent experiments.

3.2.4. Preparation of respective cell types for transplantation

For transplantation, MSC and FF were trypsinized. A sample of single cell suspension was stained with Trypan Blue and counted in a Neubauer Hemocytometer. The remaining single cell suspension was washed twice and resuspended in PBS to yield a final concentration of $0.6x10^5$ cells/µl.

3.2.5. Surgical procedures

For all surgical procedures animals were anaesthetized using a cocktail of ketamine, xylazine and acepromazine as described above.

3.2.5.1. Cervical dorsal column transection

Lesioning of the dorsal columns was performed using a tungsten wire knife (Kopf Instruments, Tujunga, USA) to transect the dorsal columns bilaterally, thereby interrupting the dorsal component of the corticospinal projection and a portion of the rostrally projecting proprioceptive dorsal sensory pathway. Rats were fixed in a spinal stereotactic unit (Kopf Instruments, Tujunga, CA), skin and muscles were incised and a laminectomy was performed at C3 level. A small dura incision was made. For C3 lesions, the tungsten wire knife was stereotactically positioned 0.6 mm to the left of midline and lowered to a depth of 1.1 mm (for illustration see Figure 1). The tip of the wire was extruded from the device, forming a 2.25 mm-wide wire arch, that was then raised 2 mm to lesion the corticospinal tract (CST) bilaterally. To ensure complete interruption (rather than strechting) of corticospinal axons, a 500 µm-wide glass pipette was tightly compressed against the wire loop under microscopic guidance.

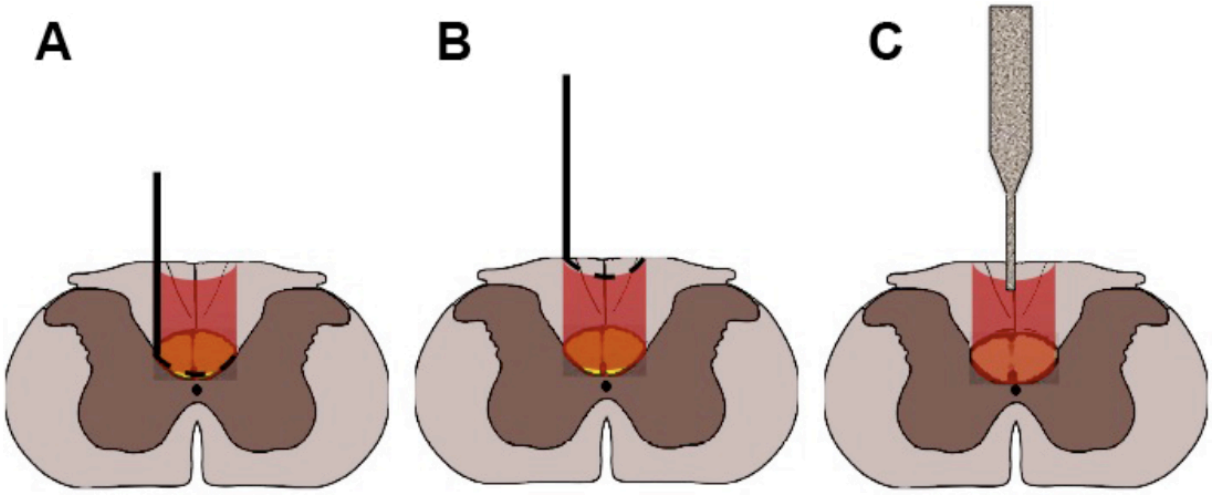


Figure 1: Schematic representation of the Cervical dorsal column transection model
The crossed dorsal components of CST axons of rats contain about 95% of the descending axons, whereas an ipsilateral ventral component contains less than 5% of all CST axons. (A) In this SCI model, a dura incision is made and the wire knife device is stereotactically lowered into the spinal cord parenchyma. At the correct depth, the tungsten wire is extruded, forming a wire arch below the dorsal component of the corticospinal projections. (B) Afterwards, the wire knife device is rised up until the tip of the wire is visible, transecting the dorsal columns bilaterally and interrupting the dorsal component of the corticospinal projections and a portion of the rostrally projecting proprioceptive dorsal sensory pathway. The wire arch is then retracted back into the wire knife device, and the instrument is removed from the cord, thereby leaving the dura intact. (C) Directly following the lesion, cell-grafts can be injected under stereotactic guidance into the lesioned area through a pulled glass micropipette.

A large proportion of the overlying dorsal funicular sensory ascending projection was also lesioned by the procedure. Afterwards the wire arch was retracted back into the knife device and the instrument was removed from the spinal cord. This lesion is highly localized and reproducible, permitting precise characterization of local responses to injury and associations of defined axonal populations to the injury milieu[46,153,154].

3.2.5.2. Cell transplantation into the injured spinal cord

After stereotactically guided transection of the dorsal CST with a tungsten wire knife at cervical level C3, a total volume of 2 µl cell suspension containing a) $0.6x10^5$ MSC/µl (**MSC,** n=6 for each timepoint) or b) $0.6x10^5$ fibroblasts/µl (**FF,** n=6 for each timepoint) was injected directly into the lesion site through a pulled glass micropipette (100 µm internal diameter) using a Picospritzer Π (General Valve, Fairfield, USA). Animals receiving spinal

cord lesions without cell transplantation (**Lesion,** n=6 for each timepoint) served as controls. The lesion/implantation site was covered with gelfoam (Gelita Tampon; Braun, Germany) before readapting muscular layers and stapling the skin above the lesion site.

3.2.6. BrdU-injection and experimental design of the in vivo studies

A short course of daily BrdU-injection (50 mg/kg) was done intraperitoneally (i.p.) starting immediately after surgery until day 3 post-op. Animals were perfused on day 3 (2 hours after last BrdU-injection) to study endogenous cell proliferation (early timepoint). To determine survival of newborn cells derived from endogenous NPC, BrdU (50 mg/kg) was administered daily i.p. from day 3-10 post-op before animals were sacrificed on day 28 (late timepoint). For illustration see Figure 2.

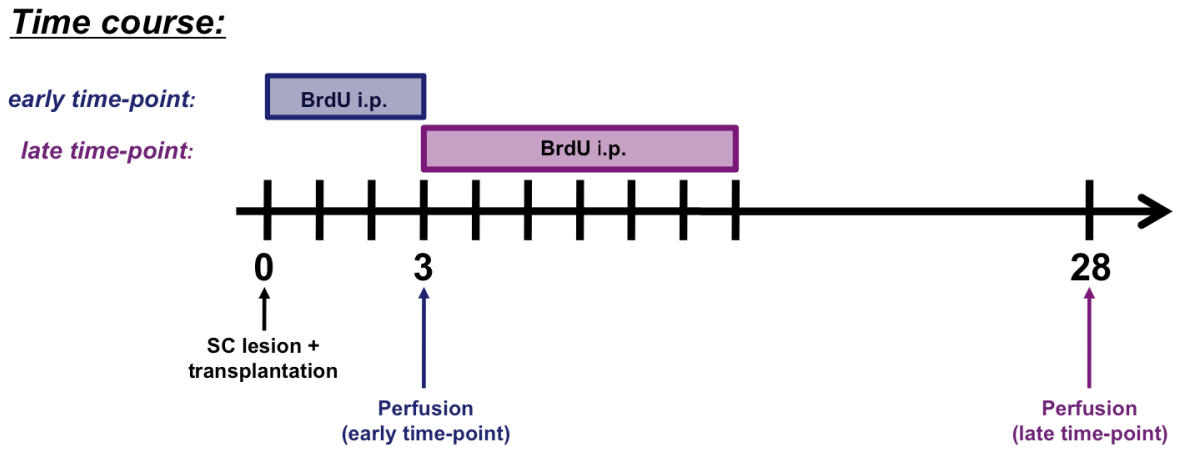


Figure 2: Schematic representation of the experimental design
All animals received a cervical wire knife dorsal column transection. Early time-point: Animals received BrdU injections intraperitoneally once a day on 4 consecutive days, starting right after the surgery. Perfusion of the rats was performed on post-op day 3. Late time-point: Rats received BrdU injections once a day for 8 consecutive days, starting on post-op day 3. Animals were perfused 4 weeks postoperatively.

3.2.7. Histology

At 3 or 28 days postoperatively, animals were transcardially perfused with 0.9% saline solution for 10 min followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. Spinal cords were dissected, post fixed overnight in 4% paraformaldehyde and cryoprotected in PBS containing 30% sucrose at 4°C. A 9 mm block of the cervical spinal cord containing the lesion center was divided into three equidistant consecutive levels termed rostral (RO), lesion center (LC), and caudal (CA), which were cut into 40 µm thick coronal cryostat sections. Three sections (one from each level) with a distance of 3 mm between each other were processed for immunohistochemistry (for illustration see Figure 3). Every seventh section was collected for Nissl staining to exactly determine the lesion/injection site and to assess the overall neuropathological changes (cystic lesion defect, tissue loss, hemorrhage). Sections mounted onto gelatine-coated coverslips were hydrated in a downward ethanol series starting with a 1:2 chloroform/ethanol mixture. Coverslips were incubated consecutively for 2 min each in ethanol 100%, 95%, 70% and 40% and for 5 min in distilled water. For the actual staining procedure coverslips were incubated in a thionine-solution for 30 seconds. After several rinses in distilled water coverslips were dehydrated consecutively for 2 min each in ethanol 40%, 70%, 80% and 95%. Finally, coverslips were incubated for 5 min in NeoClear and coverslipped with NeoMount.

All other sections were stored in 25% glycerol, 25% ethylene glycol and 50% 0.1 M sodium phosphate solution at 4°C.

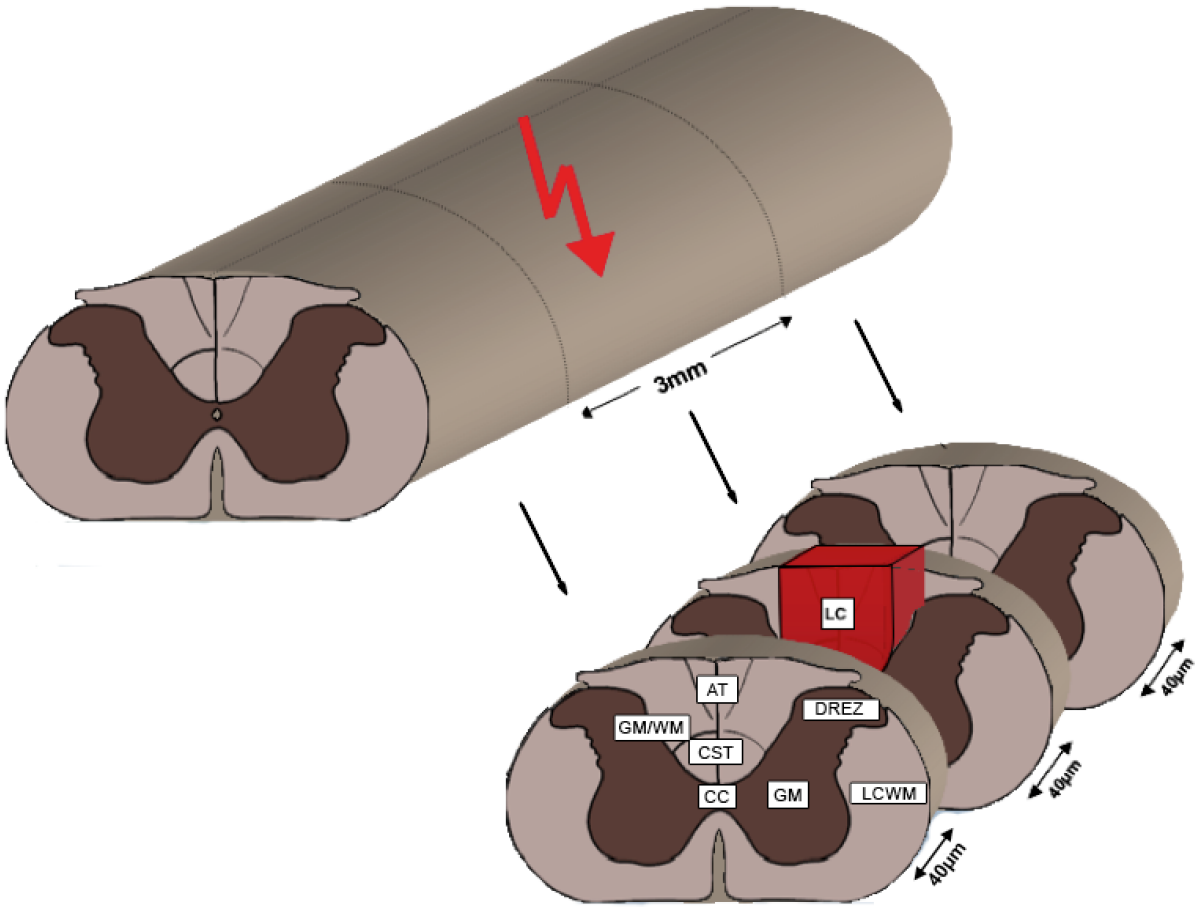


Figure 3: Schematic representation of the morphological analysis of endogenous NPC in the injured spinal cord
A 9 mm long piece of the spinal cord was cut into 40 µm thick coronal sections. Three levels (lesion center (LC), rostral (RO) and caudal (CA)) with a distance of 3 mm between each other were quantified. Within each level one field of view was quantified in up to seven different regions: 1.) central canal (CC), 2.) grey matter / white matter transition zone (GM/WM), 3.) corticospinal tract (CST), 4.) ascending tract (AT), 5.) grey matter (GM), 6.) lateral column white matter (LCWM) and 7.) dorsal root entry zone (DREZ). For quantification of the BrdU-DAB staining, all seven regions were analyzed, whereas only the first four regions (CC, GM/WM, CST and AT) were analyzed for the fluorescence staining. The red area indicates the lesion site.

3.2.8. Immunhistochemistry

3.2.8.1. Brightfield analysis

Visualization of BrdU incorporating cells with 3,3´diaminobenzidine (DAB) was processed as follows: to detect BrdU-labeled nuclei the following DNA denaturation steps preceded the incubation with a BrdU specific antibody: after rinsing in TBS, free-floating sections were incubated in 0.6% H_2O_2 for 30 min, washed again and incubated for 1h in 50% formamide/2xSSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C. Sections were rinsed

in 2xSSC, incubated for 30 min in 2 M HCl at 37°C and rinsed for 10 min in 0.1 M boric acid (pH 8.5). After rinsing in TBS, sections were blocked with a solution composed of TBS, 0.2% fish skin gelatine, 1% bovine serum albumin and 0.1% Trinton X-100 (only for intracellular antigens) for 1h. This buffer was also used during incubation with the rat anti-BrdU (1:500) primary antibody over night at 4°C. For chromogenic immunodetection, sections were washed extensively and further incubated with a biotin-conjugated species-specific secondary antibody, donkey anti-rat biotin conjugated (1:500), followed by a peroxidase-avidin complex solution from the Vectastain Elite ABC kit. Sections were developed in TBS containing 0.25 mg/ml 3,3´diaminobenzidine (DAB), 0.01% (v/v) H_2O_2, and 0.04% /w/v $NiCl_2$. Sections were mounted on gelatine-coated slides, air-dried, dehydrated and coverslipped with Neo-Mount.

3.2.8.2. Fluorescence analysis

Triple labeling immunfluorescence techniques were performed with free floating sections to assess the differentiation pattern of endogenous NPC in vivo. Sections were washed in TBS, blocked with a solution of TBS, 3% donkey serum and 0.1% Triton X-100 (only for intracellular antigens) for 1h and afterwards incubated with the primary antibodies overnight at 4°C on a rotating platform. For detection of BrdU-labeled nuclei, the following DNA denaturation steps preceded the incubation with rat anti-BrdU antibody: after rinsing in TBS, sections were incubated for 1h in 50% formamide/2xSSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C. Sections were rinsed in 2xSSC, incubated for 30 min in 2 M HCl at 37°C and rinsed for 10 min in 0.1 M boric acid (pH 8.5).

The following primary antibodies were used: rat anti-BrdU (1:500) to label newly created cells, rabbit anti-GFAP for astroglia (1:1000) and mouse anti-Adenomatous polyposis coli (APC) for oligodendrocytes (1:500). The following day, sections were rinsed and incubated with fluorescein (donkey anti-mouse IgG Alexa Fluor 488, 1:1000), rhodamine X (donkey

anti-rat RHOX, 1:500) and Cy5 (donkey anti-rabbit CY5, 1:500) conjugated donkey secondary antibodies for 2h. After a final rinsing step in TBS, sections were mounted onto glass slides and coverslipped with Prolong Antifade.

3.2.9. Microscopical analysis

Brightfield micrographs of DAB-stained sections were obtained with a Leica fluorescence microscope (Leica, Wetzlar, Germany) connected to a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Sterling Heights, USA). Quantitative analysis of BrdU-DAB labeled cells was performed using a 40x objective lens. BrdU-immunoreactive nuclei were counted in one section out of each spinal cord level (RO, LC or CA) with a distance of 3mm between each level. Within each section BrdU-immunoreactive nuclei were quantified in up to seven different regions in one field of view (see Figure 3): central canal (CC), grey/white matter transition zone (GM/WM), corticospinal tract (CST), ascending tract (AT), grey matter (GM), lateral column white matter (LCWM) and dorsal root entry zone (DREZ). Graphs are expressed as the total number of cells detected in all seven regions and in all three sections.

Immunhistochemical analysis was performed using a Leica confocal fluorescence microscope TCS-NT (Leica, Wetzlar, Germany) equipped with a 40x PL APO oil objective (1.25 numeric aperture). Co-localization of BrdU-labeled cells with the individual differentiation markers was determined by analyzing between 30-35 adjacent optical sections through the z-axis of 40µm thick coronal sections. Co-localization was confirmed, once the differentiation marker was spatially associated to BrdU nuclear labeling through subsequent optical sections in the z-axis.

The *in vivo* differentiation pattern of endogenous spinal cord NPC was quantified in three sections from 3 different levels of spinal cord: LC, RO and CA. Within each section co-localization of differentiation markers with BrdU was quantified in one field of view out of 4 different regions (for illustration see Figure 3): CC, GM/WM, CST and AT. The total

number of BrdU positive cells in each field of view was counted and correlated to the number of BrdU positive cells co-localizing with the respective differentiation marker (GFAP, APC). As APC immunoreactivity is present both in oligodendrocytes and a subset of astrocytes[155,156], only cells immunoreactive for BrdU and APC - not for GFAP - were classified as oligodendroglia. Cells immunorective for BrdU and GFAP were counted as astroglia.

3.2.10. Statistical analysis

Statistical analysis of the in vitro experiments was performed using one-way analysis of variances (ANOVA) followed by Tukey´s Multiple Comparision Test for multiple group comparisons (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). The in vivo part was analyzed using parametric one-way analysis of variances (ANOVA) and Dunn´s Multiple Comparison Test post-hoc (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using PRISM4 software (GraphPad Software, San Diego, USA).

4. RESULTS

4.1. IN VITRO

4.1.1. MSC and FF derived soluble factors induce oligodendroglial differentiation in NPC in vitro

As previously shown, either direct co-culture with MSC or incubation with soluble factors derived from MSC significantly enhance oligodendroglial differentiation in NPC derived from the adult hippocampus and the SVZ[138,147,148]. To evaluate whether this pro-oligodendrogliogenic effect is also present in NPC derived from the spinal cord, NPC from the hippocampus (HC), the subventricular zone (SVZ) and the spinal cord (SC) were cultured in MSC-CM for one week and subsequently analyzed for the differentiation markers GFAP and MBP (for illustration see Figure 4A-F). As control groups primary FF-CM was added alone or in combination with MSC-CM to NPC cultures. NPC grown with alpha-MEM 10% FBS served as control.

The capacity of MSC-CM and FF-CM to induce the expression of oligodendroglial markers in NPC derived from the SVZ, the HC or the SC in comparison to control medium (α-MEM-10% FBS) was determined in vitro (Figure 4 and 5). Independent of the origin of NPC (SVZ, HC, SC) FF-CM was the strongest promoter of MBP expressing cells (74.2% ± 4.0 in SVZ, 76.6% ± 0.5 in HC and 73.4% ± 3.4 in SC) followed by MSC-CM+FF-CM (55.7% ± 3.2 in SVZ, 55.8% ± 1.3 in HC and 54.5% ± 10.4 in SC), MSC-CM (27.9% ± 8.6 in SVZ, 43.6% ± 12.8 in HC and 50.1% ± 0.9 in SC) and alpha-MEM (2.9% ± 2.5 in SVZ, 4.5% ± 1.4 in HC and 2.4% ± 0.9 in SC). At the same time FF-CM reduced the number of GFAP expressing cells (6.5% ± 0.1 in SVZ, 7.8% ± 3.2 in HC and 10.7% ± 0.9 in SC) more than MSC-CM+FF-CM (20.7% ± 3.3 in SVZ, 25.2% ± 5.4 in HC and 12.8% ± 1.6 in SC), MSC-CM (44.6% ± 7.4 in SVZ, 58.1% ± 8.1 in HC and 27.5% ± 2.0 in SC) and alpha-MEM (80.6% ± 1.8 in SVZ, 80.1% ± 7.2 in HC and 78.0% ± 2.0 in SC).

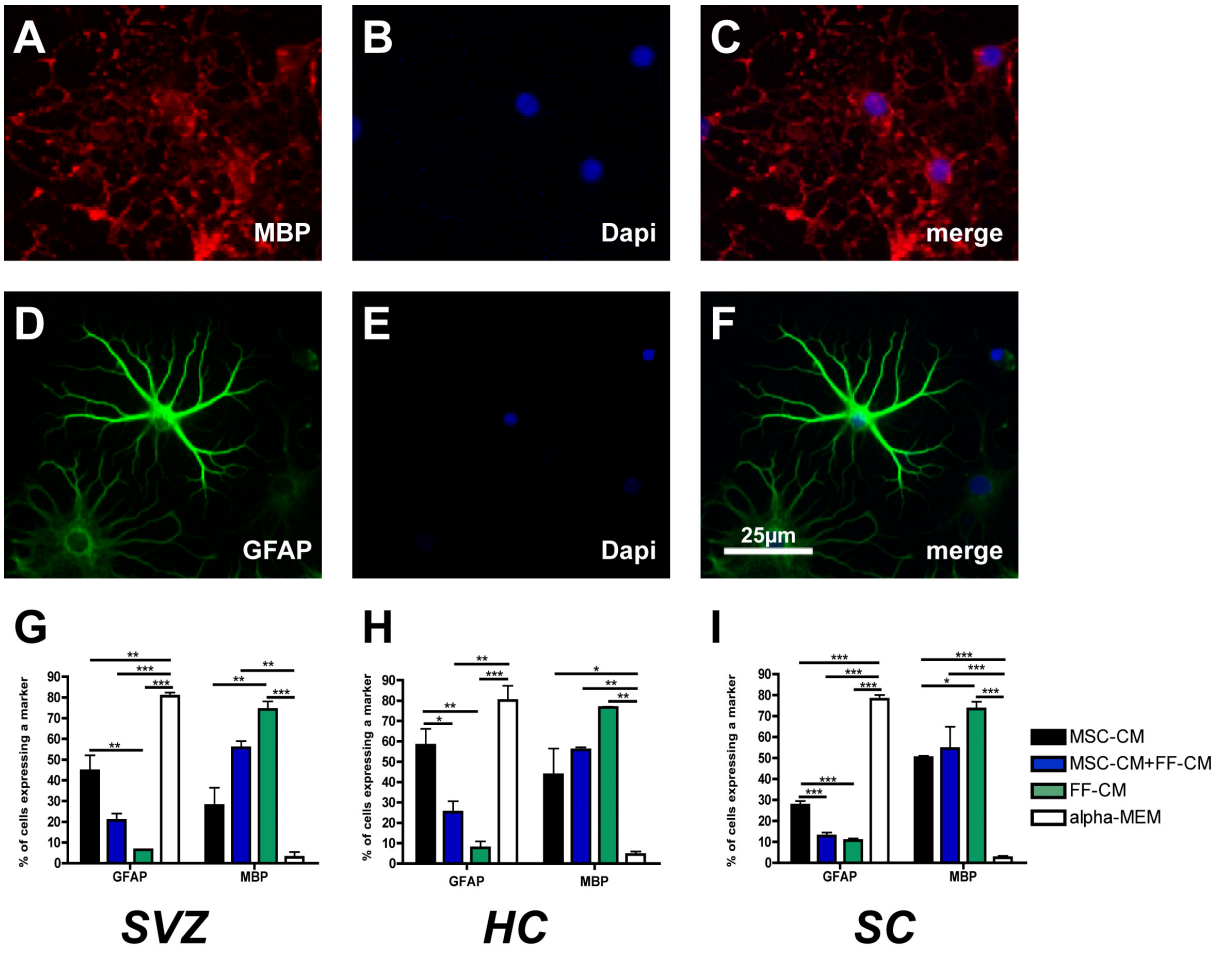


Figure 4: MSC-CM and FF-CM promote oligodendrogenesis of SVZ-, HC- as well as SC-derived NPC *in vitro*

Example of NPC (SC) expressing MBP (A-C) or GFAP (D-F). The respective marker (A,D) and DAPI counterstain (B,E) were overlayed (C,F). Quantitative analysis of MBP/GFAP expression in adult NPC derived from the SVZ (G), HC (H) or SC (I) incubated with either MSC-CM, MSC-CM+FF-CM, FF-CM or alpha-MEM. Five hundred to 1.000 GFP-positive cells (NPC) were analyzed in randomly chosen fields. Experiments were done in triplicate. Scale bar: 25 µm. Averages are expressed with their standard error of the mean (SEM). One-way analysis of variances (ANOVA) followed by Tukey´s Multiple Comparision Test for multiple group comparisons was used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

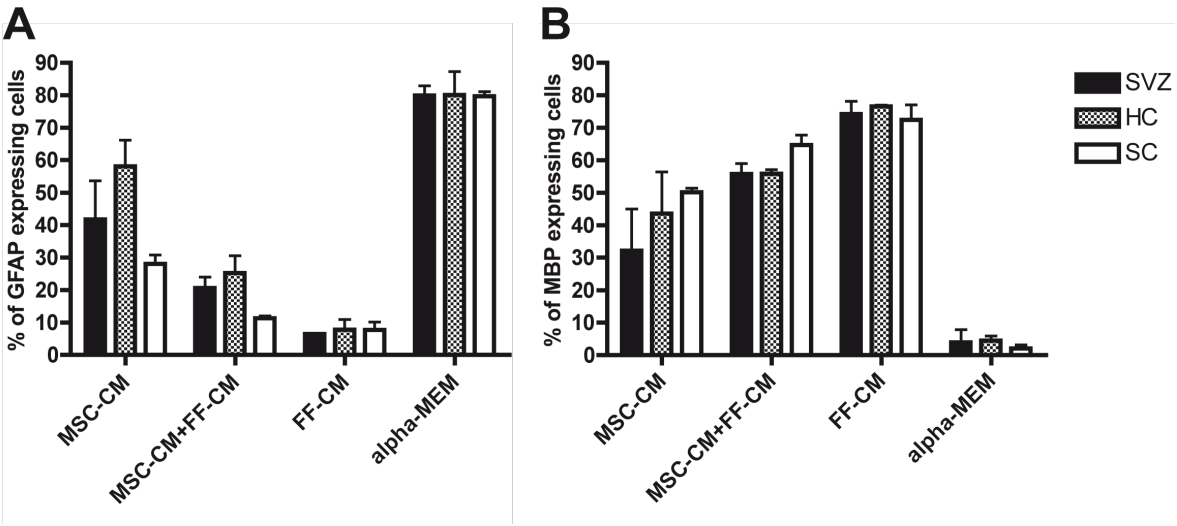


Figure 5: The origin of NPC does not influence their differentiation potential *in vitro*
Quantitative analysis of GFAP (A) and MBP (B) expression in adult NPC derived from the SVZ, HC or SC incubated with either MSC-CM, MSC-CM+FF-CM, FF-CM or alpha-MEM. Five hundred to 1.000 GFP-positive cells (NPC) were analyzed in randomly chosen fields. Experiments were done in triplicate. Averages are expressed with their standard error of the mean (SEM). One-Way ANOVA was used for statistical analysis.

In summary, FF-CM represents the most effective conditioned medium to promote oligodendroglial differentiation (MBP expression) at the expense of astroglial differentiation, irrespective of the NPC origin. Unexpectedly, this pro-oligodendrogenic effect was less pronounced after incubation of NPC with MSC-CM.

4.2. IN VIVO

4.2.1. MSC and fibroblast grafts replace the cystic lesion defect in the injured spinal cord

The key question of this study was to determine, whether MSC and FF derived soluble factors would be able to induce oligodendroglial differentiation in endogenous NPC after transplantation into the injured rat spinal cord. Therefore MSC as well as FF were transplanted directly into the lesion site of an acute spinal cord injury. MSC or FF were transplanted as cell suspension directly into the lesion site immediately after a cervical dorsal column transection. Animals receiving spinal cord lesions without cell transplantation (Lesion) served as controls. Four weeks postoperatively, the injury site displayed the typical round-triangular lesion shaped cavity in the dorsal half of the spinal cord as described previously[130] (for illustration see Figure 6).

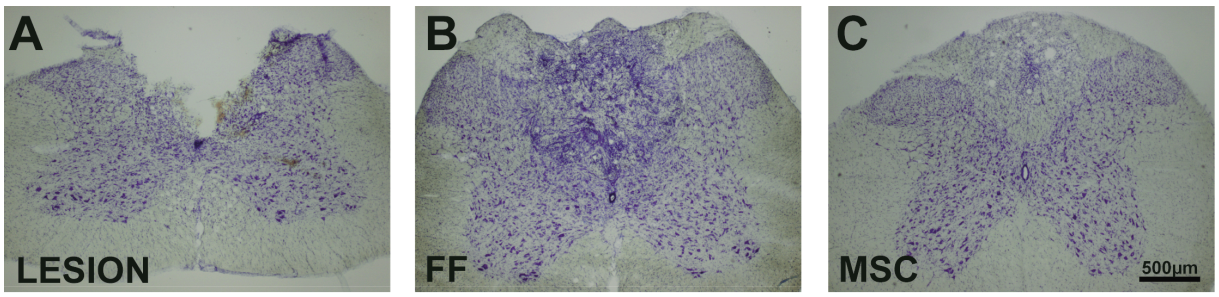


Figure 6: Replacement of the cystic lesion defect
Four weeks following a cervical wire knife dorsal column transection a typical triangular shaped cystic lesion defect develops in animals with spinal cord lesion only (A), whereas FF grafts (B) and MSC grafts (C) replace the lesion defect completely. A-C: coronal Nissl stained sections, dorsal top; scale bar: 500µm.

Both MSC and FF grafts integrated well into the host parenchyma and replaced the cystic lesion defect completely. There were no signs of uncontrolled graft proliferation or tumor formation detectable in any of the animals.

4.2.2. MSC transplantation does not alter the proliferation and survival of newborn cells after spinal cord injury

The experimental design of the in vivo studies was divided into two different time courses in order to investigate proliferation (**early timepoint**) and survival (**late timepoint**) of newborn cells after SCI (see Figure 2).

In order to examine the proliferation rate of newborn cells within the spinal cord, BrdU was administered intraperitoneally once a day for 4 consecutive days, starting right after the animals received a dorsal column transection followed by immediate cell transplantation of either MSC (**MSC**) or fibroblasts (**FF**), whereas animals receiving lesion only served as control (**Lesion**). Three days postoperatively (**early timepoint**), animals were perfused, processed for BrdU-DAB-staining and analyzed thereafter as described above (for illustration see Figure 3). Quantitative analysis of BrdU-labeled cells revealed that the number of BrdU-positive cells was significantly increased in the FF (288.8 ± 38.7) versus MSC (165.8 ± 20.4) graft recipients 3 days post-injury (Figure 7A-C, G).

In order to investigate the survival of newborn cells **(late timepoint)**, animals received intraperitoneal BrdU-injections daily starting at day 3 until day 10 postoperatively. Four weeks postoperatively BrdU positive cells were quantified. In contrast to the early time point, the number of surviving newborn cells was significantly reduced in FF grafts (113.0 ± 17.8) compared to MSC grafts (252.2 ± 71.9) and lesion only animals (279.5 ± 49.4). This effect was seen in all sections analyzed (RO, LC, CA) as well as in the total of the three sections (Figure 7D-F, H).

Taken together, MSC transplantation into the injured cervical rat spinal cord did not alter cell proliferation and survival of newborn cells, whereas FF grafting decreased survival of newborn cells (late timepoint). Furthermore FF transplantation increased the number of proliferating cells (early timepoint) compared to MSC grafting but not to lesion only.

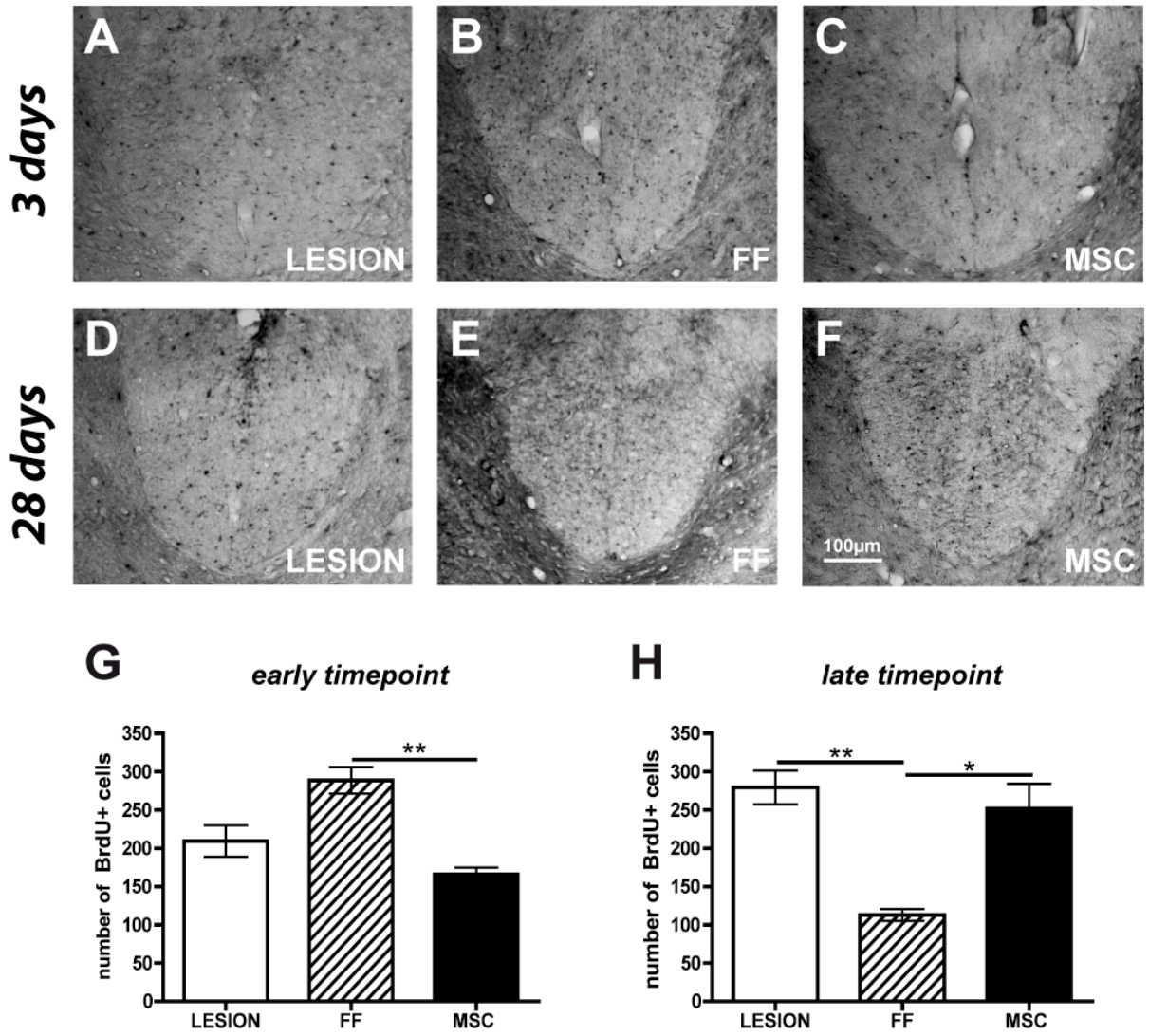


Figure 7: MSC do not alter the proliferation or survival of endogenous cells after spinal cord injury
Proliferation (A-C) and survival (D-F) of newborn cells after SCI; Brightfield images of coronal BrdU-DAB stained sections of the three differnt groups: Lesion (A,D), FF (B,E) and MSC (C,F); dorsal top; scale bar: 100µm. (G, H) Quantitative analysis of BrdU-positive cells at the early (G) respectively late timepoint (H). The amount of BrdU positive cells is expressed as total of the three analyzed sections (LC, RO, CA). Averages are expressed with their standard error of the mean (SEM). Parametric one-way ANOVA (with Dunn´s Multiple Comparison Test post-hoc) was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$.

4.2.3. MSC enhance oligodendroglial differentiation in endogenous NPC already within 3 days after SCI

The extent of astro- and oligodendroglial differentiation of cells, which incorporated BrdU within 3 days after SCI was investigated with laser confocal microscopy. The co-localization of BrdU- and APC- (for oligodendroglial cells) or GFAP-immunoreactivity (for astroglial cells) was analyzed (Figure 8)

Already at the 3-day timepoint MSC grafts increased the number of APC immunoreactive cells (BrdU+/APC+/GFAP-) compared to FF grafts and lesion only (MSC 5.2% ± 0.9; FF 0.9% ± 0.2; Lesion 1.2% ± 0.2; Figure 8I). Conversely, the number of newborn cells expressing GFAP was significantly reduced after MSC transplantation (51.6% ± 1.2) compared to the FF transplantation group (59.9% ± 2.1; Figure 8J).

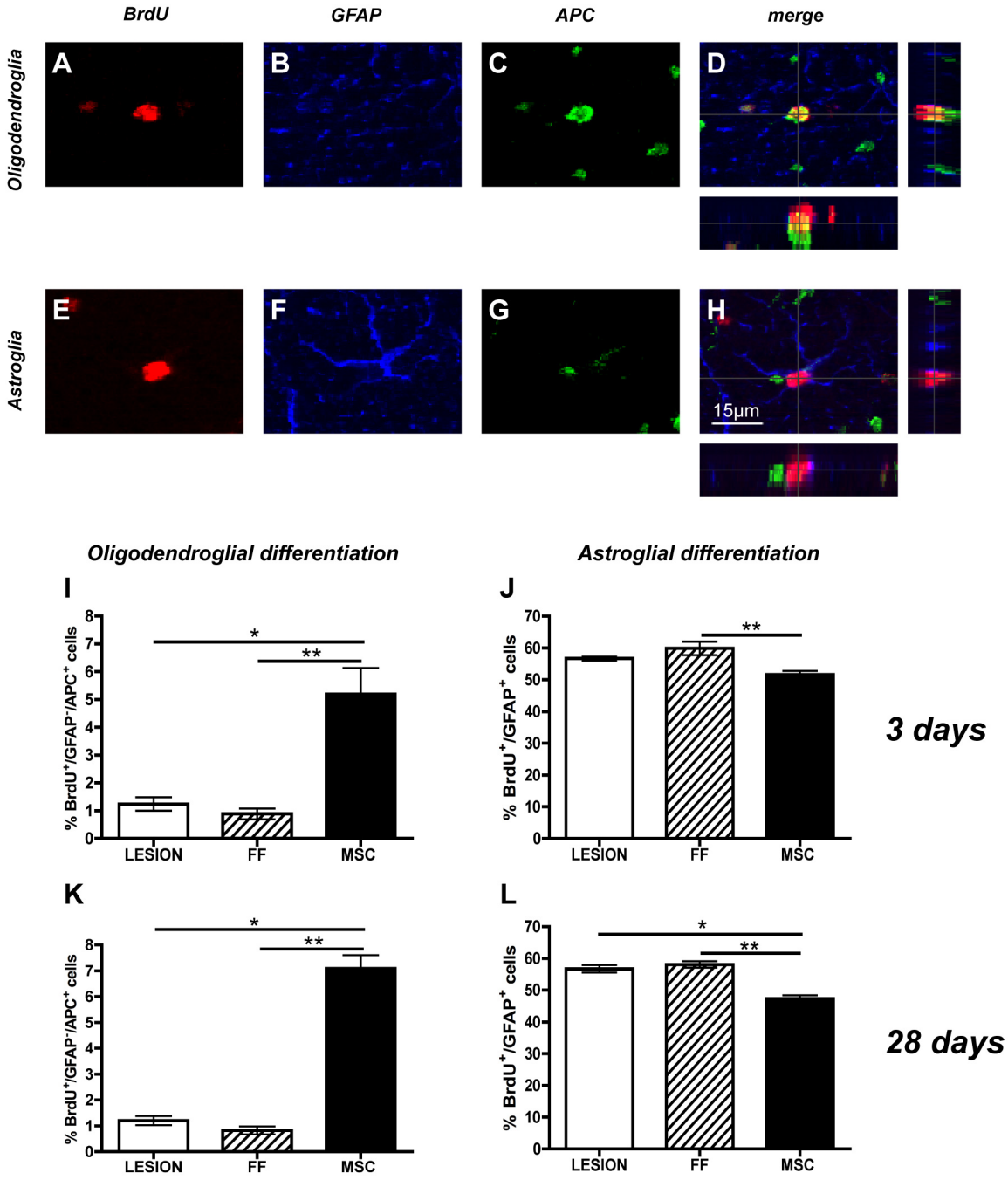


Figure 8: Influence of MSC grafting on glial differentiation of endogenous progenitor cells
The co-localization of (A) BrdU- and (C) APC-immunoreactivity together with the lack of (B) GFAP expression indicated oligodendroglial differentiation (MSC group); (D) Overlay A-C; Co-localization of (E) BrdU and (F) GFAP with or without (G) APC expression indicated astroglial differentiation (Lesion group); (H) Overlay E-G; scale bar: 15µm.
Quantitative analysis of the expression of neural differentiation markers in endogenous NPC three days respectively four weeks after cell grafting. Graphs show the percentage of oligodendroglial- (I,K) and astroglial- (J,L) differentiation. The percentages of BrdU positive oligodendrocytes and astrocytes are shown as the total number of all regions/sections analyzed.
Averages are expressed with their standard error of the mean (SEM). Parametric one-way ANOVA (with Dunn´s Multiple Comparison Test post-hoc) was used for statistical analysis; * $p < 0.05$; ** $p < 0.01$.

4.2.4. MSC grafts shift the differentiation of endogenous progenitor cells towards oligodendroglia four weeks after SCI

At 4 weeks postoperatively, MSC grafts significantly increased the number of BrdU positive cells expressing the oligodendroglial marker APC compared to FF grafts and the lesion only group (MSC 7.1% ± 0.5; FF 0.8% ± 0.2; Lesion 1.5% ± 0.2; Figure 8K). Conversely, MSC significantly decreased the number of BrdU positive cells expressing GFAP in comparison to FF grafts and the lesion only group (MSC 47.3% ± 1.0; FF 58.1% ± 1.0; Lesion 56.7% ± 1.1; Figure 8L). Taken together, MSC grafts shifted the differentiation pattern of endogenous NPC towards oligodendroglia at the expense of astroglial cells 28 days postoperatively, whereas FF grafts failed to recapitulate the pro-oligodendrogenic effect observed in vitro.

5. DISCUSSION

Based on recent findings that oligodendroglial differentiation can be strongly increased in vitro by co-cultivating adult NPC with bone-marrow derived MSC or MSC-CM[147,148], the aim of the present study was to evaluate if this effect can be recapitulated in an in vivo model of SCI. Indeed, this study demonstrates that MSC grafted into the acutely injured rat spinal cord promote oligodendroglial differentiation in endogenous NPC as prerequisite for enhanced remyelination. Over the last years the activation of endogenous progenitor cells emerged as an upcoming approach for neural repair[157]. Since different groups have described an increase of endogenous neural stem cell proliferation following SCI[98,158-164] the recruitment of this cell population and its modulation towards an oligodendroglial fate represents a promising strategy for tissue repair and restoration of neurological function.

The demonstration of an increased number of APC expressing cells after MSC transplanation, which identifies mature oligodendroglial differentiation[138,156,165-169], is only a first step towards structural and functional improvement after spinal cord injury. Above enhancement of oligodendrogliogenesis MSC have been attributed beneficial effects on remyelination in different animal models of neurological disorders[170-172]. The next step is to proof enhanced remyelination beyond spontaneous remyelination occurring in the injured mammalian CNS. Up to date there are very few studies, which clearly show enhanced remyelination induced by either boosting endogenous or exogenous (stem cell transplantation) oligodendroglial replacement[132,173-176]. Therefore it is difficult to analyze functional recovery, in particular to link the observed functional improvement to the observed structural repair – remyelination[128]. The majority of studies describe moderate improvement in locomotor function usually with a semiquantitative locomotor rating score (e.g. the Basso, Beattie and Bresnahan (BBB) locomotor scale method)[106,177], which is unspecific in terms of the structural correlate and questionable in terms of its clinical relevance. Once all these preclinical issues are solved the final question is the one regarding the clinical relevance of

such a remyelination approach. There is evidence from small animals and human SCI that uninjured, but permanently demyelinated axons exist following spinal cord injury. It has yet to be demonstrated that successful remyelination of this undefined quantity of axons is sufficient to promote meaningful functional improvement in humans. More likely, the remyelination promoting capacity of MSC needs to be combined with axon regrowth promoting strategies (see below).

Increased olidgodendrogliogenesis, indicated by APC expression, was already observed at 3 days post-injury and consecutive MSC grafting. In vitro and in vivo studies confirm this time line. The number of progenitor cells expressing markers for mature oligodendroglia (APC, MBP, GalC) is already enhanced at 3 days after exposure to MSC-CM in vitro[147-149,178]. In vivo (spinal cord demyelination model), a shift from immature oligodendrocyte precursor cells (NG2+/BrdU+) towards mature oligodendrocytes (APC+/BrdU+) between 2 and 7 days after a single shot BrdU administration has been described[163].

Paralleling previous in vitro findings[147,148] renewal of astroglia expressing GFAP was reduced at 4 weeks post-operatively, suggesting that enhanced oligodendroglial replacement happens at the expense of astroglial replacement. Studies investigating cell proliferation and renewal after SCI found almost exclusively differentiation into astrocytes[179-181].

In contrast to the successful promotion of oligodendroglial replacement from endogenous progenitor pools after MSC grafting, shifting of oligodendroglial differentiation failed in a co-grafting paradigm with MSC and NPC[137,138]. Here the host environment after spinal cord injury most likely prevented proper oligodendroglial differentiation in grafted NPC. Main molecular candidates are bone morphogenetic proteins (BMP) - strong inducers of astroglial differentiaton, which are upregulated immediately after SCI[182-185]. The BMP mediated prevention of oligodendroglial differentiation is supported by in vitro findings, where MSC-CM is not strong enough to override BMP induced astroglial differentiation[138]. Of course the

question arises, why do we observe enhanced oligodendroglial replacement from endogenous progenitor pools after MSC grafting, and not a shift of oligodendroglial differentiation after MSC/NPC co-grafting? NPC grafts get fully exposed to BMP expressed after SCI at the lesion site, whereas endogenous progenitor cells become recruited continuously over time. Since BMP become downregulated within 10 days following injury, MSC derived soluble factor(s) can exert their pro-oligodendrogenic effects in endogenous progenitor cells throughout the subacute phase beyond 10 days post injury.

These findings once again underline the importance of the local microenvironment at the injury site, which influences the fate of stem/progenitor cell grafts. Previous studies confirm these findings showing that molecular cues of the host environment rather than the predifferentiation of transplanted cells determine the differentiation of grafted stem cells. The same batch of a given neural stem/progenitor cell type differentiates into neurons after grafting into neurogenic regions such as the hippocampus and exclusively into glial cells after grafting into non-neurogenic regions such as the spinal cord[46,130,186-188]. On the other hand, the origin of adult NPC does not affect the glial/neuronal differentiation pattern vitro. This supports the notion that NPC can be obtained from various regions of the CNS without affecting the yield of cells differentiated into either astroglia, oligodendroglia or neurons. If certain differentiation phenotypes are to be maintained after transplantation, in particular local cues derived from the host such as BMP need to be addressed.

MSC grafts did not interfer with cell proliferation, whereas FF grafts increased the number of BrdU positive cells within the first few days after SCI. Syngenic cell grafting in general can induce a local inflammatory response contributing to the high cell proliferation rate. It is concievable that MSC grafts with their anti-inflammatory, immune-modulating[189-193] as well as anti-apoptotic[194,195] properties counteract this proliferation of inflammatory cells thus reducing cell proliferation directly or indirectly. At 4 weeks postoperatively, the number of surviving newborn cells was reduced in the FF group suggesting that the initial stronger

inflammatory response led to increased cell death and poor cell survival in the long run. This is in contrast to former findings in the brain which showed increased endogenous progenitor cell proliferation consecutive to intracerebral/intravenous MSC injection after brain injury[196] as well as MSC grafting after stroke[197]. These contrary findings may be caused by a somehow differing predominant microenvironment conducive to cell proliferation inside the brain compared to the spinal cord. In addition it has to be mentioned that no direct comparison can be drawn for the present study as the underlying experimental designs significantly differed from each other (used animals, injury model, cell graft administration, timecourse of BrdU-labeling and perfusion).

Differential effects of MSC-CM in respect to the origin of NPC – HC, SVZ or spinal cord – were investigated more closely but did not reveal any differences. This is confirmed by a recent report, which did not reveal any differences in terms of neuronal versus glial differentiation in adult SVZ or spinal cord derived NPC[198]. This finding is potentially clinically relevant since the accessibility of "donor" regions within the CNS determines, whether an autologous adult stem cell based therapy is feasible or not.

Surprisingly, strong pro-oligodendrogenic effects were also observed in FF-CM. Since MSC and FF belong to mesenchymal tissue derived from the mesoderm, it is conceivable that the identical soluble factor is responsible for these effects[199,200]. As the combination of MSC-CM and FF-CM did not produce higher numbers of cells displaying oligodendroglial differentiation compared to MSC-CM or FF-CM incubation alone, the yet unidentified soluble factor(s) might at least in part act on the same signaling pathway[138]. Alternatively, FF produce this factor in higher concentrations or express an additional factor, which also supports oligodendroglial fate determination. According to our in vitro data it is conceivable that fibroblasts may express this unidentified factor at a higher concentration[201] or even express additional factors to promote oligodendroglial differentiation. Overall, several candidate factors have been investigated previously, which do not have a significant impact

on oligodendroglial differentation: fibroblast-growth factor-2 (FGF-2), brain-derived-neurotrophic-factor (BDNF), vascular endothelian growth factor (VEGF), ciliary-neurotrophic factor (CNTF), nerve growth factor (NGF), neurotrophin-3 (NT-3), interleukin-6 (IL-6), insulin-like growth factor-1 (IGF-1), Noggin[147-149]. Of course, the identification of these pro-oligodendrogenic factors(s) will be important to gain further mechanistic insights into the process of oligodendroglial differentiation. This may in the end allow to introduce factors, which are efficient enough to overcome host derived cues opposing oligodendroglial differentiation.

Besides remyelination as a potential repair mechanism of MSC grafts, MSC grafts have been widely investigated in small animal models of SCI. Besides neuroprotective effects after SCI, MSC have been described to attenuate glial scar formation[202] and to promote axonal regeneration[135,203-205] with concomitant functional recovery[151,170,206,207]. MSC grafts replaced the cystic lesion defect completely confirming previous reports, which demonstrated long-term survival and cyst replacement by MSC grafts[130,135,138,208]. Cyst replacement is a key prerequisite for axon regrowth across the lesion site. Furthermore, MSC can be genetically modified to overexpress and release growth factors such as BDNF, NGF or NT-3 locally at the lesion site to augment axonal growth[135,172,209]. Recently, the combination of NT-3 overexpressing MSC and genetic modification of the host spinal cord adjacent to the lesion site to create a chemotrophic gradient has been shown to induce axon regrowth across the lesion into the target region within the caudal brainstem with reinnervation of previous target neurons[210]. However, restoration of proper nerve conduction could not be detected yet, which is most likely due to a lack of myelination of regenerating axons. It is conceivable that effects of MSC-CM next to regenerating axons in the host spinal cord might promote remyelination and consecutively restored nerve conduction.

The relevance of regenerative strategies aiming for remyelination to induce functional recovery is underscored by two different cell-based therapies, which were translated into

phase I clinical trials. In the first approach, embryonic derived stem cells predifferentiated into OPC have been shown to promote remyelination and functional improvement in rat SCI models[176,211]. Unfortunately, the respective clinical trial in acute SCI patients was stopped after four patients were treated for yet unknown reasons. However, side effects or complications of the transplantation procedure were not reported. In another study, fetal human brain derived stem cells are investigated in another phase I clinical trial, after preclinical studies in rats and mice reported remyelination and functional improvement after experimental SCI and demyelination[175,212-214]. This clinical trial, which started in 2011, is still ongoing[128].

6. CONCLUSION

Transplantation of MSC into the acute injured spinal cord is sufficient to enhance oligodendroglial differentiation of endogenous spinal cord progenitor cells up to one month post-injury. Results from the present study and previously published data confirm that underyling mechanisms are based on (a) soluble factor(s) secreted by MSC, which create a specific microenvironment supporting oligodendrogliogenesis at the lesion site. The identification of these pro-oligodendrogenic factors will be important to gain more mechanistic insights in the interactions between MSC and the endogenous progenitor cell population within the spinal cord to further boost oligodendroglial differentiation and eventually remyelination with subsequent functional recovery. Before the translation into clinical trial can be considered clear and unequivocal MSC induced remyelination needs to be confirmed. Furthermore, functional recovery including neurophysiology (restoration of nerve conduction) and behavioral effects (e.g recovery of locomotion) need to be assessed in appropriate animal models such as contusion SCI, which show pronounced demyelination and resemble the pathophysiology of human SCI as close as possible.

7. ABBREVIATIONS

Alpha-MEM	Alpha Minimum Essential Medium
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
ASIA	American Spinal Injury Association
AT	Ascending Tract
BDNF	Brain Derived Neurotrophic Factor
BMP	Bone-morphogenetic-protein
BrdU	5´-Bromo-2´-Desoxyuridine
BSA	Bovine Serum Albumin
CA	Caudal
CC	Central Canal
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CPS	Cryo Protect Solution
CSPG	Chondroitin Sulfate Proteoglycan(s)
CST	Corticospinal tract
Cy5	Cyanine 5
DAB	3,3´-Diaminobenzidine
DAPI	4,6´-Diamidino-2-phenylindole
Dk	Donkey
DMEM	Dulbecco´s Modified Eagle Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco´s Phosphate Buffered Saline
DREZ	Dorsal Root Entry Zone
EGF	Epidermal Growth Factor
eGFP	Enhanced Green Fluorescent Protein
FBS	Fetal Bovine Serum
FF	Fibroblast(s)
FF-CM	Fibroblast-Conditioned Medium
FGF	Fibroblast Growth Factor
FSBG	Fish Skin Gelatine Buffer
GalC	Galactocerebroside
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GM	Grey Matter
GM/WM	Grey Matter/White Matter transition zone
Gt	Goat
HBSS	Hank´s Balanced Salt Solution
HC	Hippocampus
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin, isoform G
IL-6	Interleukin-6

LC	Lesion Center
LCWM	Lateral Column White Matter
MAG	Myelin-Associated Glycoprotein
Map2ab	Microtubule Associated Protein 2ab
MBP	Myelin Basic Protein
Ms	Mouse
MSC	Mesenchymal Stem Cell(s)
MSC-CM	Mesenchymal Stem Cell-Conditioned Medium
NB	Neurobasal
NG2	Chondroitin Sulfate Proteoglycan
NGF	Nerve growth factor
NPC	Neural stem/progenitor cell(s)
NT	Neurotrophin
OMgp	Oligodendrocyte Myelin Glycoprotein
OPC	Oligodendroglial Precursor Cell(s)
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
Rb	Rabbit
Rhox	Rhodamine X
RO	Rostral
Rt	Rat
SC	Spinal Cord

SCI	Spinal Cord Injury
SEM	Standard Error of the Mean
SVZ	Subventricular Zone
TBS	Tris-Buffered Saline
VEGF	Vascular Endothelian Growth Factor
WM	White Matter
ZNS	Zentrales Nervensystem

8. REFERENCES

1. Barnabe-Heider, F. & Frisen, J. Stem cells for spinal cord repair. *Cell Stem Cell* 3, 16-24 (2008).

2. Kakulas, B.A. The applied neuropathology of human spinal cord injury. *Spinal Cord* 37, 79-88 (1999).

3. Kakulas, B.A. A review of the neuropathology of human spinal cord injury with emphasis on special features. *J Spinal Cord Med* 22, 119-124 (1999).

4. Hulsebosch, C.E. Recent advances in pathophysiology and treatment of spinal cord injury. *Adv Physiol Educ* 26, 238-255 (2002).

5. van den Berg, M.E., Castellote, J.M., Mahillo-Fernandez, I. & de Pedro-Cuesta, J. Incidence of spinal cord injury worldwide: a systematic review. *Neuroepidemiology* 34, 184-192; discussion 192 (2010).

6. Chiu, W.T.*, et al.* Review paper: epidemiology of traumatic spinal cord injury: comparisons between developed and developing countries. *Asia Pac J Public Health* 22, 9-18 (2010).

7. Sekhon, L.H. & Fehlings, M.G. Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine (Phila Pa 1976)* 26, S2-12 (2001).

8. N.S.C.I.S.C. Spinal Cord Injury Statistics Updated June 2009. http://www.fscip.org/facts.htm

9. Wyndaele, M. & Wyndaele, J.J. Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? *Spinal Cord* 44, 523-529 (2006).

10. Karacan, I.*, et al.* Traumatic spinal cord injuries in Turkey: a nation-wide epidemiological study. *Spinal Cord* 38, 697-701 (2000).

11. Connor, D.F.*, et al.* Juvenile maladaptive aggression: a review of prevention, treatment, and service configuration and a proposed research agenda. *J Clin Psychiatry* 67, 808-820 (2006).

12. Tator, C.H., Duncan, E.G., Edmonds, V.E., Lapczak, L.I. & Andrews, D.F. Changes in epidemiology of acute spinal cord injury from 1947 to 1981. *Surg Neurol* 40, 207-215 (1993).

13. DeVivo, M.J., Stover, S.L. & Black, K.J. Prognostic factors for 12-year survival after spinal cord injury. *Arch Phys Med Rehabil* 73, 156-162 (1992).

14. Tator, C.H. Spine-spinal cord relationships in spinal cord trauma. *Clin Neurosurg* 30, 479-494 (1983).

15. Querschnittslähmung - Leitlinien für Diagnostik und Therapie in der Neurologie. *Georg Thieme Verlag Stuttgart,* 4. überarbeitete Auflage 2008, S. 654 ff.

16. Tator, C.H. Update on the pathophysiology and pathology of acute spinal cord injury. *Brain Pathol* 5, 407-413 (1995).

17. Tator, C.H. Experimental and clinical studies of the pathophysiology and management of acute spinal cord injury. *J Spinal Cord Med* 19, 206-214 (1996).

18. Tator, C.H. Biology of neurological recovery and functional restoration after spinal cord injury. *Neurosurgery* 42, 696-707; discussion 707-698 (1998).

19. Bunge, R.P., Puckett, W.R., Becerra, J.L., Marcillo, A. & Quencer, R.M. Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. *Adv Neurol* 59, 75-89 (1993).

20. Bunge, R.P., Puckett, W.R. & Hiester, E.D. Observations on the pathology of several types of human spinal cord injury, with emphasis on the astrocyte response to penetrating injuries. *Adv Neurol* 72, 305-315 (1997).

21. Tator, C.H. & Fehlings, M.G. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *J Neurosurg* 75, 15-26 (1991).

22. Casha, S., Yu, W.R. & Fehlings, M.G. Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat. *Neuroscience* 103, 203-218 (2001).

23. Crowe, M.J., Bresnahan, J.C., Shuman, S.L., Masters, J.N. & Beattie, M.S. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat Med* 3, 73-76 (1997).

24. de la Torre, J.C. Spinal cord injury. Review of basic and applied research. *Spine (Phila Pa 1976)* 6, 315-335 (1981).

25. Goodman, J.H., Bingham, W.G., Jr. & Hunt, W.E. Ultrastructural blood-brain barrier alterations and edema formation in acute spinal cord trauma. *J Neurosurg* 44, 418-424 (1976).

26. Hsu, C.Y.*, et al.* Vascular permeability in experimental spinal cord injury. *J Neurol Sci* 70, 275-282 (1985).

27. Senter, H.J. & Venes, J.L. Loss of autoregulation and posttraumatic ischemia following experimental spinal cord trauma. *J Neurosurg* 50, 198-206 (1979).

28. Tator, C.H. Review of experimental spinal cord injury with emphasis on the local and systemic circulatory effects. *Neurochirurgie* 37, 291-302 (1991).

29. Tator, C.H. & Koyanagi, I. Vascular mechanisms in the pathophysiology of human spinal cord injury. *J Neurosurg* 86, 483-492 (1997).

30. Agrawal, S.K. & Fehlings, M.G. Mechanisms of secondary injury to spinal cord axons in vitro: role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+ exchanger, and the Na(+)-Ca2+ exchanger. *J Neurosci* 16, 545-552 (1996).

31. Young, W. & Koreh, I. Potassium and calcium changes in injured spinal cords. *Brain Res* 365, 42-53 (1986).

32. Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D. & Seligman, M.L. The free radical pathology and the microcirculation in the major central nervous system disorders. *Acta Physiol Scand Suppl* 492, 91-119 (1980).

33. Dykens, J.A. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated CA2+ and Na+: implications for neurodegeneration. *J Neurochem* 63, 584-591 (1994).

34. Hall, E.D. & Braughler, J.M. Free radicals in CNS injury. *Res Publ Assoc Res Nerv Ment Dis* 71, 81-105 (1993).

35. Agrawal, S.K. & Fehlings, M.G. Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. *J Neurosci* 17, 1055-1063 (1997).

36. Demediuk, P., Daly, M.P. & Faden, A.I. Effect of impact trauma on neurotransmitter and nonneurotransmitter amino acids in rat spinal cord. *J Neurochem* 52, 1529-1536 (1989).

37. Faden, A.I. & Simon, R.P. A potential role for excitotoxins in the pathophysiology of spinal cord injury. *Ann Neurol* 23, 623-626 (1988).

38. Raisman, G. Myelin inhibitors: does NO mean GO? *Nat Rev Neurosci* 5, 157-161 (2004).

39. Yiu, G. & He, Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci* 7, 617-627 (2006).

40. Schmitt, A.B.*, et al.* Major histocompatibility complex class II expression by activated microglia caudal to lesions of descending tracts in the human spinal cord is not associated with a T cell response. *Acta Neuropathol* 100, 528-536 (2000).

41. Bethea, J.R.*, et al.* Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats. *J Neurotrauma* 16, 851-863 (1999).

42. Dusart, I. & Schwab, M.E. Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. *Eur J Neurosci* 6, 712-724 (1994).

43. Popovich, P.G., Wei, P. & Stokes, B.T. Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J Comp Neurol* 377, 443-464 (1997).

44. Wada, K., Chatzipanteli, K., Busto, R. & Dietrich, W.D. Role of nitric oxide in traumatic brain injury in the rat. *J Neurosurg* 89, 807-818 (1998).

45. Kao, t. & chang, L.W. The mechanism of spinal cord cavitation following spinal cord transection. *J Neurosurg* 46, 197-209 (1977).

46. Vroemen, M., Aigner, L., Winkler, J. & Weidner, N. Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. *Eur J Neurosci* 18, 743-751 (2003).

47. Kao, C.C., Chang, L.W. & Bloodworth, J.M., Jr. Axonal regeneration across transected mammalian spinal cords: an electron microscopic study of delayed microsurgical nerve grafting. *Exp Neurol* 54, 591-615 (1977).

48. Kao, C.C., Chang, L.W. & Bloodworth, J.M., Jr. Electron microscopic observations of the mechanisms of terminal club formation in transected spinal cord axons. *J Neuropathol Exp Neurol* 36, 140-156 (1977).

49. Willerth, S.M. & Sakiyama-Elbert, S.E. Cell therapy for spinal cord regeneration. *Adv Drug Deliv Rev* 60, 263-276 (2008).

50. Fawcett, J.W. & Asher, R.A. The glial scar and central nervous system repair. *Brain Res Bull* 49, 377-391 (1999).

51. Reier, P.J. & Houle, J.D. The glial scar: its bearing on axonal elongation and transplantation approaches to CNS repair. *Adv Neurol* 47, 87-138 (1988).

52. McKerracher, L. Spinal cord repair: strategies to promote axon regeneration. *Neurobiol Dis* 8, 11-18 (2001).

53. Rolls, A., Shechter, R. & Schwartz, M. The bright side of the glial scar in CNS repair. *Nat Rev Neurosci* 10, 235-241 (2009).

54. Fok-Seang, J.*, et al.* An analysis of astrocytic cell lines with different abilities to promote axon growth. *Brain Res* 689, 207-223 (1995).

55. Silver, J. & Miller, J.H. Regeneration beyond the glial scar. *Nat Rev Neurosci* 5, 146-156 (2004).

56. Bush, T.G.*, et al.* Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 23, 297-308 (1999).

57. Faulkner, J.R.*, et al.* Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci* 24, 2143-2155 (2004).

58. McKerracher, L.*, et al.* Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* 13, 805-811 (1994).

59. Prinjha, R.*, et al.* Inhibitor of neurite outgrowth in humans. *Nature* 403, 383-384 (2000).

60. Bomze, H.M., Bulsara, K.R., Iskandar, B.J., Caroni, P. & Skene, J.H. Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat Neurosci* 4, 38-43 (2001).

61. Bonilla, I.E., Tanabe, K. & Strittmatter, S.M. Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. *J Neurosci* 22, 1303-1315 (2002).

62. Becker, T.*, et al.* Readiness of zebrafish brain neurons to regenerate a spinal axon correlates with differential expression of specific cell recognition molecules. *J Neurosci* 18, 5789-5803 (1998).

63. Bulsara, K.R., Iskandar, B.J., Villavicencio, A.T. & Skene, J.H. A new millenium for spinal cord regeneration: growth-associated genes. *Spine (Phila Pa 1976)* 27, 1946-1949 (2002).

64. Lindsay, R.M., Wiegand, S.J., Altar, C.A. & DiStefano, P.S. Neurotrophic factors: from molecule to man. *Trends Neurosci* 17, 182-190 (1994).

65. Schnell, L., Schneider, R., Kolbeck, R., Barde, Y.A. & Schwab, M.E. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* 367, 170-173 (1994).

66. Kobayashi, N.R.*, et al.* BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talpha1-tubulin mRNA expression, and promote axonal regeneration. *J Neurosci* 17, 9583-9595 (1997).

67. Davies, A.M. The role of neurotrophins in the developing nervous system. *J Neurobiol* 25, 1334-1348 (1994).

68. Giehl, K.M. & Tetzlaff, W. BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. *Eur J Neurosci* 8, 1167-1175 (1996).

69. Gledhill, R.F., Harrison, B.M. & McDonald, W.I. Demyelination and remyelination after acute spinal cord compression. *Exp Neurol* 38, 472-487 (1973).

70. Fleming, J.C.*, et al.* The cellular inflammatory response in human spinal cords after injury. *Brain* 129, 3249-3269 (2006).

71. Beattie, M.S.*, et al.* Endogenous repair after spinal cord contusion injuries in the rat. *Exp Neurol* 148, 453-463 (1997).

72. Kim, D.H., Vaccaro, A.R., Henderson, F.C. & Benzel, E.C. Molecular biology of cervical myelopathy and spinal cord injury: role of oligodendrocyte apoptosis. *Spine J* 3, 510-519 (2003).

73. Li, Y., Field, P.M. & Raisman, G. Death of oligodendrocytes and microglial phagocytosis of myelin precede immigration of Schwann cells into the spinal cord. *J Neurocytol* 28, 417-427 (1999).

74. Emery, E.*, et al.* Apoptosis after traumatic human spinal cord injury. *J Neurosurg* 89, 911-920 (1998).

75. Totoiu, M.O. & Keirstead, H.S. Spinal cord injury is accompanied by chronic progressive demyelination. *J Comp Neurol* 486, 373-383 (2005).

76. Buss, A.*, et al.* Gradual loss of myelin and formation of an astrocytic scar during Wallerian degeneration in the human spinal cord. *Brain* 127, 34-44 (2004).

77. Kakulas, B.A. Neuropathology: the foundation for new treatments in spinal cord injury. *Spinal Cord* 42, 549-563 (2004).

78. Beattie, M.S., Farooqui, A.A. & Bresnahan, J.C. Review of current evidence for apoptosis after spinal cord injury. *J Neurotrauma* 17, 915-925 (2000).

79. Beattie, M.S., Hermann, G.E., Rogers, R.C. & Bresnahan, J.C. Cell death in models of spinal cord injury. *Prog Brain Res* 137, 37-47 (2002).

80. Yong, C.*, et al.* Apoptosis in cellular compartments of rat spinal cord after severe contusion injury. *J Neurotrauma* 15, 459-472 (1998).

81. Blight, A.R. Delayed demyelination and macrophage invasion: a candidate for secondary cell damage in spinal cord injury. *Cent Nerv Syst Trauma* 2, 299-315 (1985).

82. Blight, A.R. & Decrescito, V. Morphometric analysis of experimental spinal cord injury in the cat: the relation of injury intensity to survival of myelinated axons. *Neuroscience* 19, 321-341 (1986).

83. Shuman, S.L., Bresnahan, J.C. & Beattie, M.S. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. *J Neurosci Res* 50, 798-808 (1997).

84. McEwen, M.L. & Springer, J.E. A mapping study of caspase-3 activation following acute spinal cord contusion in rats. *J Histochem Cytochem* 53, 809-819 (2005).

85. Siegenthaler, M.M., Tu, M.K. & Keirstead, H.S. The extent of myelin pathology differs following contusion and transection spinal cord injury. *J Neurotrauma* 24, 1631-1646 (2007).

86. Kakulas, B.A. Pathology of spinal injuries. *Cent Nerv Syst Trauma* 1, 117-129 (1984).

87. Nashmi, R., Jones, O.T. & Fehlings, M.G. Abnormal axonal physiology is associated with altered expression and distribution of Kv1.1 and Kv1.2 K+ channels after chronic spinal cord injury. *Eur J Neurosci* 12, 491-506 (2000).

88. Nashmi, R. & Fehlings, M.G. Mechanisms of axonal dysfunction after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels. *Brain Res Brain Res Rev* 38, 165-191 (2001).

89. Gledhill, R.F., Harrison, B.M. & McDonald, W.I. Pattern of remyelination in the CNS. *Nature* 244, 443-444 (1973).

90. Salgado-Ceballos, H.*, et al.* Spontaneous long-term remyelination after traumatic spinal cord injury in rats. *Brain Res* 782, 126-135 (1998).

91. Bunge, M.B., Bunge, R.P. & Ris, H. Ultrastructural study of remyelination in an experimental lesion in adult cat spinal cord. *J Biophys Biochem Cytol* 10, 67-94 (1961).

92. Bunge, R.P., Bunge, M.B. & Rish. Electron microscopic study of demyelination in an experimentally induced lesion in adult cat spinal cord. *J Biophys Biochem Cytol* 7, 685-696 (1960).

93. Ffrench-Constant, C. & Raff, M.C. Proliferating bipotential glial progenitor cells in adult rat optic nerve. *Nature* 319, 499-502 (1986).

94. Franklin, R.J. & Ffrench-Constant, C. Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci* 9, 839-855 (2008).

95. Shields, S.A., Gilson, J.M., Blakemore, W.F. & Franklin, R.J. Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. *Glia* 28, 77-83 (1999).

96. Woodruff, R.H. & Franklin, R.J. Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: a comparative study. *Glia* 25, 216-228 (1999).

97. Jeffery, N.D. & Blakemore, W.F. Remyelination of mouse spinal cord axons demyelinated by local injection of lysolecithin. *J Neurocytol* 24, 775-781 (1995).

98. Reynolds, R.*, et al.* The response of NG2-expressing oligodendrocyte progenitors to demyelination in MOG-EAE and MS. *J Neurocytol* 31, 523-536 (2002).

99. Wolswijk, G. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J Neurosci* 18, 601-609 (1998).

100. Kotter, M.R., Li, W.W., Zhao, C. & Franklin, R.J. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J Neurosci* 26, 328-332 (2006).

101. Miller, R.H. Contact with central nervous system myelin inhibits oligodendrocyte progenitor maturation. *Dev Biol* 216, 359-368 (1999).

102. Syed, Y.A.*, et al.* Inhibition of oligodendrocyte precursor cell differentiation by myelin-associated proteins. *Neurosurg Focus* 24, E5 (2008).

103. Kakulas, A. The applied neurobiology of human spinal cord injury: a review. *Paraplegia* 26, 371-379 (1988).

104. Kakulas, B.A. The clinical neuropathology of spinal cord injury. A guide to the future. *Paraplegia* 25, 212-216 (1987).

105. Noble, L.J. & Wrathall, J.R. Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. *Exp Neurol* 103, 34-40 (1989).

106. Basso, D.M., Beattie, M.S. & Bresnahan, J.C. Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. *Exp Neurol* 139, 244-256 (1996).

107. Blight, A.R. Morphometric analysis of a model of spinal cord injury in guinea pigs, with behavioral evidence of delayed secondary pathology. *J Neurol Sci* 103, 156-171 (1991).

108. Jones, L.L., Oudega, M., Bunge, M.B. & Tuszynski, M.H. Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. *J Physiol* 533, 83-89 (2001).

109. Bonner, J.F., Blesch, A., Neuhuber, B. & Fischer, I. Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. *J Neurosci Res* 88, 1182-1192 (2010).

110. Oudega, M. & Hagg, T. Neurotrophins promote regeneration of sensory axons in the adult rat spinal cord. *Brain Res* 818, 431-438 (1999).

111. Ye, J.H. & Houle, J.D. Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. *Exp Neurol* 143, 70-81 (1997).

112. Bradbury, E.J. & McMahon, S.B. Spinal cord repair strategies: why do they work? *Nat Rev Neurosci* 7, 644-653 (2006).

113. Mason, M.R., Lieberman, A.R. & Anderson, P.N. Corticospinal neurons up-regulate a range of growth-associated genes following intracortical, but not spinal, axotomy. *Eur J Neurosci* 18, 789-802 (2003).

114. Liu, B.P., Fournier, A., GrandPre, T. & Strittmatter, S.M. Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. *Science* 297, 1190-1193 (2002).

115. Chen, M.S.*, et al.* Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403, 434-439 (2000).

116. Fawcett, J.W. Overcoming inhibition in the damaged spinal cord. *J Neurotrauma* 23, 371-383 (2006).

117. Schwab, J.M.*, et al.* Experimental strategies to promote spinal cord regeneration - an integrative perspective. *Prog Neurobiol* 78, 91-116 (2006).

118. Li, S., Kim, J.E., Budel, S., Hampton, T.G. & Strittmatter, S.M. Transgenic inhibition of Nogo-66 receptor function allows axonal sprouting and improved locomotion after spinal injury. *Mol Cell Neurosci* 29, 26-39 (2005).

119. Lee, J.K.*, et al.* Reassessment of corticospinal tract regeneration in Nogo-deficient mice. *J Neurosci* 29, 8649-8654 (2009).

120. Zheng, B.*, et al.* Lack of enhanced spinal regeneration in Nogo-deficient mice. *Neuron* 38, 213-224 (2003).

121. Kim, J.E., Li, S., GrandPre, T., Qiu, D. & Strittmatter, S.M. Axon regeneration in young adult mice lacking Nogo-A/B. *Neuron* 38, 187-199 (2003).

122. Simonen, M.*, et al.* Systemic deletion of the myelin-associated outgrowth inhibitor Nogo-A improves regenerative and plastic responses after spinal cord injury. *Neuron* 38, 201-211 (2003).

123. Smith-Thomas, L.C.*, et al.* Increased axon regeneration in astrocytes grown in the presence of proteoglycan synthesis inhibitors. *J Cell Sci* 108 (Pt 3), 1307-1315 (1995).

124. Jones, L.L., Sajed, D. & Tuszynski, M.H. Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition. *J Neurosci* 23, 9276-9288 (2003).

125. Prang, P.*, et al.* The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. *Biomaterials* 27, 3560-3569 (2006).

126. Coutts, M. & Keirstead, H.S. Stem cells for the treatment of spinal cord injury. *Exp Neurol* 209, 368-377 (2008).

127. Kim, B.G., Hwang, D.H., Lee, S.I., Kim, E.J. & Kim, S.U. Stem cell-based cell therapy for spinal cord injury. *Cell Transplant* 16, 355-364 (2007).

128. Sandner, B.*, et al.* Neural stem cells for spinal cord repair. *Cell Tissue Res* (2012).

129. Tetzlaff, W.*, et al.* A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury. *J Neurotrauma* (2010).

130. Pfeifer, K., Vroemen, M., Blesch, A. & Weidner, N. Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury. *Eur J Neurosci* 20, 1695-1704 (2004).

131. Pfeifer, K.*, et al.* Autologous adult rodent neural progenitor cell transplantation represents a feasible strategy to promote structural repair in the chronically injured spinal cord. *Regen Med* 1, 255-266 (2006).

132. Karimi-Abdolrezaee, S., Eftekharpour, E., Wang, J., Morshead, C.M. & Fehlings, M.G. Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. *J Neurosci* 26, 3377-3389 (2006).

133. Weidner, N., Blesch, A., Grill, R.J. & Tuszynski, M.H. Nerve growth factor-hypersecreting Schwann cell grafts augment and guide spinal cord axonal growth and remyelinate central nervous system axons in a phenotypically appropriate manner that correlates with expression of L1. *J Comp Neurol* 413, 495-506 (1999).

134. Grill, R.J., Blesch, A. & Tuszynski, M.H. Robust growth of chronically injured spinal cord axons induced by grafts of genetically modified NGF-secreting cells. *Exp Neurol* 148, 444-452 (1997).

135. Lu, P., Jones, L.L. & Tuszynski, M.H. BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. *Exp Neurol* 191, 344-360 (2005).

136. Hofstetter, C.P.*, et al.* Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nat Neurosci* 8, 346-353 (2005).

137. Sandner, B. Co-transplantation of adult neural progenitor cells with cells of mesenchymal origin for oligodendroglia replacement after spinal cord injury. *Society for Neuroscience 38th annual meeting (Washington D.C., USA)* (2008).

138. Sandner, B. Co-Transplantation of adult neural stem/progenitor cells together with cells of mesenchymal origin into the injured spinal cord. *Dissertation - Universität Regensburg* (2010).

139. Blakemore, W.F. & Crang, A.J. The use of cultured autologous Schwann cells to remyelinate areas of persistent demyelination in the central nervous system. *J Neurol Sci* 70, 207-223 (1985).

140. Honmou, O., Felts, P.A., Waxman, S.G. & Kocsis, J.D. Restoration of normal conduction properties in demyelinated spinal cord axons in the adult rat by transplantation of exogenous Schwann cells. *J Neurosci* 16, 3199-3208 (1996).

141. Lu, J. & Ashwell, K. Olfactory ensheathing cells: their potential use for repairing the injured spinal cord. *Spine (Phila Pa 1976)* 27, 887-892 (2002).

142. Akiyama, Y., Lankford, K., Radtke, C., Greer, C.A. & Kocsis, J.D. Remyelination of spinal cord axons by olfactory ensheathing cells and Schwann cells derived from a transgenic rat expressing alkaline phosphatase marker gene. *Neuron Glia Biol* 1, 47-55 (2004).

143. Radtke, C.*, et al.* Remyelination of the nonhuman primate spinal cord by transplantation of H-transferase transgenic adult pig olfactory ensheathing cells. *FASEB J* 18, 335-337 (2004).

144. Sasaki, M., Lankford, K.L., Zemedkun, M. & Kocsis, J.D. Identified olfactory ensheathing cells transplanted into the transected dorsal funiculus bridge the lesion and form myelin. *J Neurosci* 24, 8485-8493 (2004).

145. Enzmann, G.U., Benton, R.L., Talbott, J.F., Cao, Q. & Whittemore, S.R. Functional considerations of stem cell transplantation therapy for spinal cord repair. *J Neurotrauma* 23, 479-495 (2006).

146. Parr, A.M., Tator, C.H. & Keating, A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. *Bone Marrow Transplant* 40, 609-619 (2007).

147. Rivera, F.J.*, et al.* Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. *Stem Cells* 24, 2209-2219 (2006).

148. Rivera, F.J., *et al.* Mesenchymal stem cells promote oligodendroglial differentiation in hippocampal slice cultures. *Cell Physiol Biochem* 24, 317-324 (2009).

149. Rivera, F.J., *et al.* Oligodendrogenesis of adult neural progenitors: differential effects of ciliary neurotrophic factor and mesenchymal stem cell derived factors. *J Neurochem* 107, 832-843 (2008).

150. Wachs, F.P., *et al.* High efficacy of clonal growth and expansion of adult neural stem cells. *Lab Invest* 83, 949-962 (2003).

151. Hofstetter, C.P., *et al.* Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proc Natl Acad Sci U S A* 99, 2199-2204 (2002).

152. Tuszynski, M.H., *et al.* Fibroblasts genetically modified to produce nerve growth factor induce robust neuritic ingrowth after grafting to the spinal cord. *Exp Neurol* 126, 1-14 (1994).

153. Weidner, N., Grill, R.J. & Tuszynski, M.H. Elimination of basal lamina and the collagen "scar" after spinal cord injury fails to augment corticospinal tract regeneration. *Exp Neurol* 160, 40-50 (1999).

154. Weidner, N., Ner, A., Salimi, N. & Tuszynski, M.H. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. *Proc Natl Acad Sci U S A* 98, 3513-3518 (2001).

155. McTigue, D.M., Wei, P. & Stokes, B.T. Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. *J Neurosci* 21, 3392-3400 (2001).

156. Bhat, R.V., *et al.* Expression of the APC tumor suppressor protein in oligodendroglia. *Glia* 17, 169-174 (1996).

157. Bai, L., Caplan, A., Lennon, D. & Miller, R.H. Human mesenchymal stem cells signals regulate neural stem cell fate. *Neurochem Res* 32, 353-362 (2007).

158. Horky, L.L., Galimi, F., Gage, F.H. & Horner, P.J. Fate of endogenous stem/progenitor cells following spinal cord injury. *J Comp Neurol* 498, 525-538 (2006).

159. Xu, Y., Kitada, M., Yamaguchi, M., Dezawa, M. & Ide, C. Increase in bFGF-responsive neural progenitor population following contusion injury of the adult rodent spinal cord. *Neurosci Lett* 397, 174-179 (2006).

160. Zai, L.J. & Wrathall, J.R. Cell proliferation and replacement following contusive spinal cord injury. *Glia* 50, 247-257 (2005).

161. Yamamoto, S., Yamamoto, N., Kitamura, T., Nakamura, K. & Nakafuku, M. Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. *Exp Neurol* 172, 115-127 (2001).

162. Di Bello, I.C., Dawson, M.R., Levine, J.M. & Reynolds, R. Generation of oligodendroglial progenitors in acute inflammatory demyelinating lesions of the rat brain stem is associated with demyelination rather than inflammation. *J Neurocytol* 28, 365-381 (1999).

163. Watanabe, M., Toyama, Y. & Nishiyama, A. Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. *J Neurosci Res* 69, 826-836 (2002).

164. Levine, J.M. & Reynolds, R. Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. *Exp Neurol* 160, 333-347 (1999).

165. Chambers, J.S. & Perrone-Bizzozero, N.I. Altered myelination of the hippocampal formation in subjects with schizophrenia and bipolar disorder. *Neurochem Res* 29, 2293-2302 (2004).

166. Tripathi, R. & McTigue, D.M. Prominent oligodendrocyte genesis along the border of spinal contusion lesions. *Glia* 55, 698-711 (2007).

167. Cai, J.*, et al.* Co-localization of Nkx6.2 and Nkx2.2 homeodomain proteins in differentiated myelinating oligodendrocytes. *Glia* 58, 458-468 (2010).

168. Cao, Q.*, et al.* Transplantation of ciliary neurotrophic factor-expressing adult oligodendrocyte precursor cells promotes remyelination and functional recovery after spinal cord injury. *J Neurosci* 30, 2989-3001 (2010).

169. Fuss, B.*, et al.* Purification and analysis of in vivo-differentiated oligodendrocytes expressing the green fluorescent protein. *Dev Biol* 218, 259-274 (2000).

170. Zhang, J.*, et al.* Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp Neurol* 195, 16-26 (2005).

171. Zhang, J.*, et al.* Bone marrow stromal cells increase oligodendrogenesis after stroke. *J Cereb Blood Flow Metab* 29, 1166-1174 (2009).

172. Lu, Z.*, et al.* Overexpression of CNTF in Mesenchymal Stem Cells reduces demyelination and induces clinical recovery in experimental autoimmune encephalomyelitis mice. *J Neuroimmunol* 206, 58-69 (2009).

173. Yasuda, A.*, et al.* Significance of remyelination by neural stem/progenitor cells transplanted into the injured spinal cord. *Stem Cells* 29, 1983-1994 (2011).

174. Cao, Q.*, et al.* Functional recovery in traumatic spinal cord injury after transplantation of multineurotrophin-expressing glial-restricted precursor cells. *J Neurosci* 25, 6947-6957 (2005).

175. Cummings, B.J.*, et al.* Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A* 102, 14069-14074 (2005).

176. Keirstead, H.S.*, et al.* Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 25, 4694-4705 (2005).

177. Basso, D.M., Beattie, M.S. & Bresnahan, J.C. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12, 1-21 (1995).

178. Steffenhagen, C.*, et al.* Mesenchymal Stem Cells Prime Proliferating Adult Neural Progenitors Toward an Oligodendrocyte Fate. *Stem Cells Dev* (2011).

179. Meletis, K.*, et al.* Spinal cord injury reveals multilineage differentiation of ependymal cells. *PLoS Biol* 6, e182 (2008).

180. Johansson, C.B.*, et al.* Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96, 25-34 (1999).

181. Mothe, A.J. & Tator, C.H. Proliferation, migration, and differentiation of endogenous ependymal region stem/progenitor cells following minimal spinal cord injury in the adult rat. *Neuroscience* 131, 177-187 (2005).

182. Matsuura, I., Taniguchi, J., Hata, K., Saeki, N. & Yamashita, T. BMP inhibition enhances axonal growth and functional recovery after spinal cord injury. *J Neurochem* 105, 1471-1479 (2008).

183. Gross, R.E.*, et al.* Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron* 17, 595-606 (1996).

184. Nakashima, K.*, et al.* BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. *Proc Natl Acad Sci U S A* 98, 5868-5873 (2001).

185. Samanta, J. & Kessler, J.A. Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. *Development* 131, 4131-4142 (2004).

186. Shihabuddin, L.S., Horner, P.J., Ray, J. & Gage, F.H. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci* 20, 8727-8735 (2000).

187. Gage, F.H.*, et al.* Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92, 11879-11883 (1995).

188. Suhonen, J.O., Peterson, D.A., Ray, J. & Gage, F.H. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* 383, 624-627 (1996).

189. Ortiz, L.A.*, et al.* Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A* 104, 11002-11007 (2007).

190. Phinney, D.G. & Prockop, D.J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair - current views. *Stem Cells* 25, 2896-2902 (2007).

191. Picard-Riera, N., Nait-Oumesmar, B. & Baron-Van Evercooren, A. Endogenous adult neural stem cells: limits and potential to repair the injured central nervous system. *J Neurosci Res* 76, 223-231 (2004).

192. Uccelli, A., Moretta, L. & Pistoia, V. Immunoregulatory function of mesenchymal stem cells. *Eur J Immunol* 36, 2566-2573 (2006).

193. Uccelli, A., Pistoia, V. & Moretta, L. Mesenchymal stem cells: a new strategy for immunosuppression? *Trends Immunol* 28, 219-226 (2007).

194. Hung, S.C., Pochampally, R.R., Chen, S.C., Hsu, S.C. & Prockop, D.J. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 25, 2363-2370 (2007).

195. Inoue, Y.*, et al.* Subretinal transplantation of bone marrow mesenchymal stem cells delays retinal degeneration in the RCS rat model of retinal degeneration. *Exp Eye Res* 85, 234-241 (2007).

196. Mahmood, A., Lu, D. & Chopp, M. Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. *Neurosurgery* 55, 1185-1193 (2004).

197. Yoo, S.W.*, et al.* Mesenchymal stem cells promote proliferation of endogenous neural stem cells and survival of newborn cells in a rat stroke model. *Exp Mol Med* 40, 387-397 (2008).

198. Kulbatski, I. & Tator, C.H. Region-specific differentiation potential of adult rat spinal cord neural stem/precursors and their plasticity in response to in vitro manipulation. *J Histochem Cytochem* 57, 405-423 (2009).

199. Haniffa, M.A., Collin, M.P., Buckley, C.D. & Dazzi, F. Mesenchymal stem cells: the fibroblasts' new clothes? *Haematologica* 94, 258-263 (2009).

200. Cappellesso-Fleury, S.*, et al.* Human fibroblasts share immunosuppressive properties with bone marrow mesenchymal stem cells. *J Clin Immunol* 30, 607-619 (2010).

201. Chen, L., Tredget, E.E., Wu, P.Y. & Wu, Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 3, e1886 (2008).

202. Caplan, A.I. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 213, 341-347 (2007).

203. Cho, J.S.*, et al.* Transplantation of mesenchymal stem cells enhances axonal outgrowth and cell survival in an organotypic spinal cord slice culture. *Neurosci Lett* 454, 43-48 (2009).

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

205. Ankeny, D.P., McTigue, D.M. & Jakeman, L.B. Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats. *Exp Neurol* 190, 17-31 (2004).

206. Chen, J., Li, Y. & Chopp, M. Intracerebral transplantation of bone marrow with BDNF after MCAo in rat. *Neuropharmacology* 39, 711-716 (2000).

207. Chopp, M.*, et al.* Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. *Neuroreport* 11, 3001-3005 (2000).

208. Vroemen, M., Caioni, M., Bogdahn, U. & Weidner, N. Failure of Schwann cells as supporting cells for adult neural progenitor cell grafts in the acutely injured spinal cord. *Cell Tissue Res* 327, 1-13 (2007).

209. Taylor, L., Jones, L., Tuszynski, M.H. & Blesch, A. Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. *J Neurosci* 26, 9713-9721 (2006).

210. Alto, L.T.*, et al.* Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. *Nat Neurosci* 12, 1106-1113 (2009).

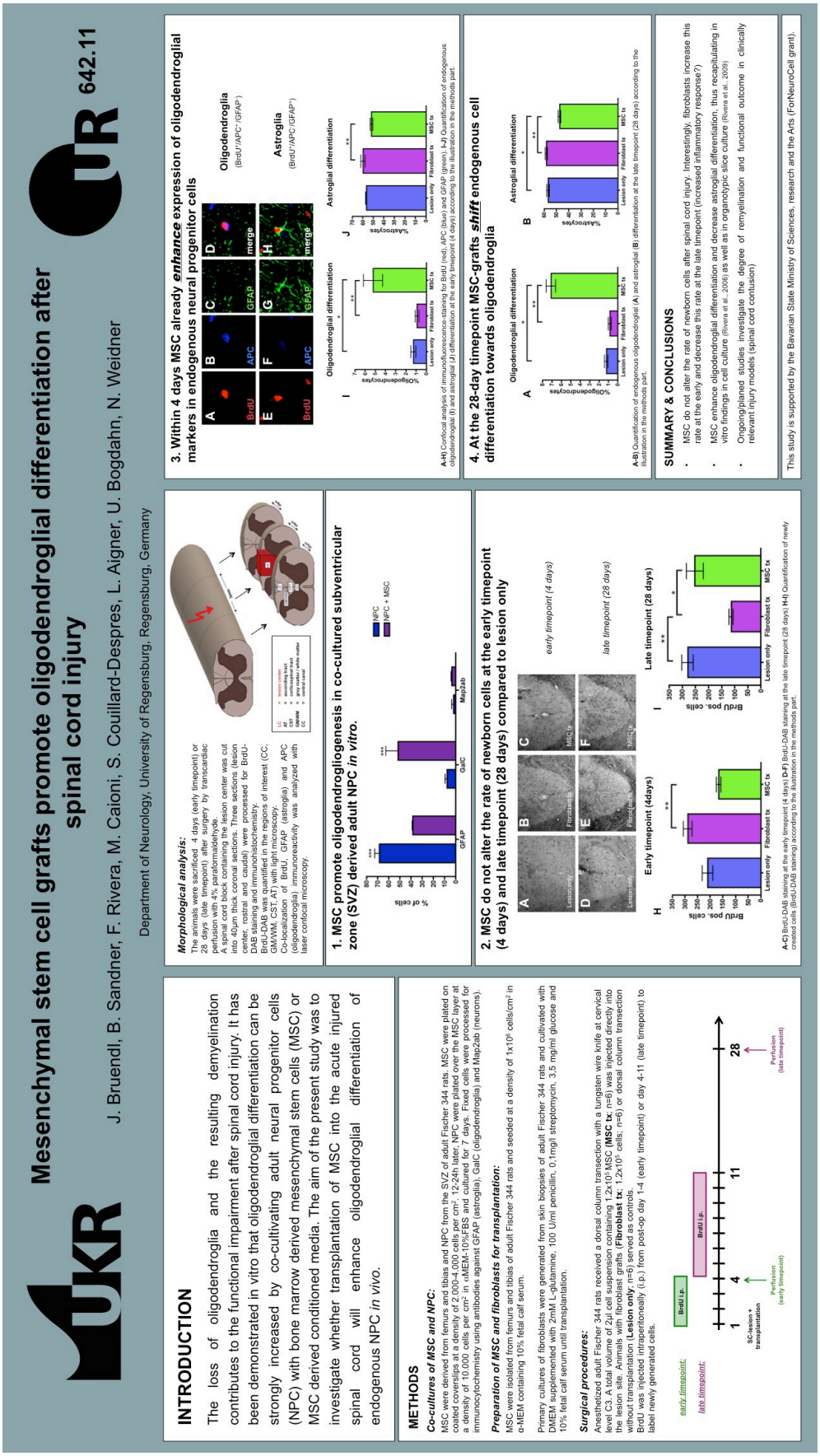
211. Sharp, J., Frame, J., Siegenthaler, M., Nistor, G. & Keirstead, H.S. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. *Stem Cells* 28, 152-163 (2010).

212. Cummings, B.J., Uchida, N., Tamaki, S.J. & Anderson, A.J. Human neural stem cell differentiation following transplantation into spinal cord injured mice: association with recovery of locomotor function. *Neurol Res* 28, 474-481 (2006).

213. Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J. & Anderson, A.J. Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. *PLoS One* 5, e12272 (2010).

214. Hooshmand, M.J.*, et al.* Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: correlation of engraftment with recovery. *PLoS One* 4, e5871 (2009).

9. ATTACHMENTS



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