HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Transplantation of stem cells from human umbilical cord blood to improve regeneration after spinal cord injury

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Jessica Schira

aus Hilden

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aus dem Labor für Molekulare Neurobiologie der Neurologischen Klinik
der Heinrich Heine Universität Düsseldorf

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To my family.

1. Summary/Zusammenfassung

1.1 Summary

Injury of the spinal cord interrupts the electrophysiological signal transduction, resulting in a long lasting loss of mobility and sensory input as well as autonomic nervous system control below the level of the lesion. Spontaneous regeneration of CNS axons fails due to massive cell death, presence of extrinsic inhibitory molecules, scar formation and the deficient growth potential of adult CNS neurons.

Stem cell therapy is a potential treatment for spinal cord injury (SCI), and different stem cell types have been grafted into animal models and humans with SCI. Due to inconsistent results, it is still an open question which stem cell type will prove to be therapeutically effective. Thus far, stem cells of human sources grafted into the spinal cord mostly included barely defined heterogeneous mesenchymal stem cell (MSC) populations derived from bone marrow or umbilical cord blood. In addition to MSC, human umbilical cord blood contains unrestricted somatic stem cells (USSC), which can be clearly discriminated from cord blood MSC by the expression of delta-like 1/preadipocyte factor 1 and a USSC-specific Hox-code. Transplantation of this well-defined human stem cell population has previously not been used as a cellular therapeutic strategy in pre-clinical animal studies treating SCI.

The focus of this study was to characterize USSC migration and neural differentiation after transplantation into a rodent model of acute SCI. Further, effects of USSC grafting on axonal regeneration, tissue preservation and functional outcome were evaluated. The present study revealed that grafting of USSC into the vicinity of a dorsal hemisection injury at thoracic level Th8 results in hepatocyte growth factor-directed migration and accumulation within the lesion area. Further, it was demonstrated that USSC transplantation results in improved axonal regeneration. Incubation of primary rat neuronal cell cultures with USSC-conditioned medium enhanced neurite outgrowth, suggesting that USSC release neurite growth promoting factors underlying the improved axonal regeneration *in vivo*. In addition, this approach revealed that USSC transplantation into an acute SCI model reduces the lesion size and leads to augmented tissue sparing. The observed tissue protection may further have contributed to enhanced locomotor recovery of USSC transplanted

rats as evaluated in a long term study with three different locomotor tests. All three locomotor tests carried out for 16 weeks post transplantation revealed that USSC-transplantation leads to a significantly and consistently improved locomotor performance. The beneficial effects were accomplished without neural differentiation or long-lasting persistence of the grafted USSC. The present data therefore reveal promising results, suggesting that transplantation of native USSC derived from human umbilical cord blood into acute spinal cord injured rats is an effective strategy to promote regenerative axon growth, tissue sparing and significant functional locomotor improvement. It would be interesting to investigate the effect of USSC transplantation on contusion injured rats, which has higher clinical relevance as this represents the most common kind of injury. Furthermore, it is currently unsolved whether USSC transplantation leads to more pronounced improvement than MSC from human umbilical cord blood. These aspects will be taken into consideration for future studies.

1.2 Zusammenfassung

Traumatische Verletzungen des Rückenmarks führen zu Unterbrechungen der elektrophysiologischen Signalweiterleitung, die distal zur Läsion den Verlust der Beweglichkeit und der Sensorik bei gleichzeitiger Funktionsstörung des vegetativen Nervensystems zur Folge hat. Eine durch die Rückenmarkverletzung bedingte Narbenbildung, massives Zellsterben sowie die Anreicherung inhibitorischer Moleküle verhindern eine spontane Regeneration der in ihrem intrinsischen Wachstumspotential ohnehin eingeschränkten Axone des adulten Zentralnervensystems.

Ein möglicher Behandlungsansatz nach Rückenmarkverletzung ist die Transplantation von Stammzellen. Bislang ist jedoch unklar, welcher Stammzelltyp einen therapeutischen Nutzen hat, da Transplantationsstudien in Tiermodellen und Patienten mit Stammzellen verschiedenen Ursprungs zu widersprüchlichen Ergebnissen führten. Bei einer Vielzahl von Transplantationsstudien mit Stammzellen menschlichen Ursprungs wurden zudem zumeist die nur unzureichend charakterisierten mesenchymalen Stammzellen verwendet, welche aus dem Knochenmark oder aus Nabelschnurrestblut gewonnen werden können. Neben mesenchymalen Stammzellen können aus Nabelschnurrestblut sogenannte unrestringierte somatische Stammzellen (USSC) isoliert werden, die durch die Expression von delta-like 1/preadipocyte factor 1 und einen USSC-spezifischen Hox-code von mesenchymalen Stammzellen zu unterscheiden sind. Ob eine Transplantation dieses Stammzelltypen nach Rückenmarkverletzung in ein klinisch-relevantes Tiermodel therapeutischen Nutzen hat, wurde bislang nicht untersucht.

Ziel der vorliegenden Arbeit war es, die Migrations- und Differenzierungs-eigenschaften von USSC nach Transplantation in akut-verletztes Rückenmark zu untersuchen. Zu diesem Zweck wurde ein bereits etabliertes Nagermodell verwendet. Des Weiteren sollte die Auswirkung der USSC-Transplantation auf axonale Regeneration, Gewebeerhalt und funktionelle Erholung analysiert werden.

In der vorliegenden Arbeit konnte gezeigt werden, dass USSC nach Transplantation in die unmittelbare Umgebung einer thorakalen dorsalen Hemisektion (thorakales Segment 8) in Abhängigkeit des Hepatozyten-

Wachstumsfaktors zur Läsion migrieren und dort akkumulieren. Ferner konnte gezeigt werden, dass die Transplantation von USSC eine axonale Regeneration fördert. Die Inkubation primärer Neuronenkulturen der Ratte mit USSC-konditioniertem Medium führte zu vermehrtem Neuritenwachstum *in vitro*. Dies lässt auf eine Ausschüttung wachstumsfördernder Faktoren schließen, die zur beobachteten axonalen Regeneration *in vivo* beitragen könnten. Zudem wurde in der vorliegenden Arbeit gezeigt, dass die Transplantation von USSC zu einer reduzierten Läsionsgröße und zu verbessertem Gewebeerhalt führt. In einer Langzeitstudie, die über einen Zeitraum von 16 Wochen nach Transplantation durchgeführt wurde, konnte mittels drei verschiedener lokomotorischer Verhaltenstests festgestellt werden, dass eine Transplantation von USSC ins verletzte Rückenmark zu konstant signifikant verbesserter Lokomotorik führt, die auf den gesteigerten Gewebeerhalt zurück zu führen sein könnte. Eine neurale Differenzierung oder dauerhafte Präsenz der USSC nach Transplantation war für die beobachteten positiven Effekte nicht nötig.

Die vorliegende Arbeit zeigt, dass die Transplantation von nativen, aus dem Nabelschnurrestblut isolierten USSC einen vielversprechenden Behandlungsansatz nach Rückenmarkverletzung darstellt. Eine Transplantation von USSC in akut-verletztes Rückenmark begünstigt demnach die axonale Regenerationsfähigkeit, den Gewebeerhalt und die lokomotorische Erholung. In einer nachfolgenden Studie ist zu klären, ob die Transplantation von USSC in ein klinisch relevanteres Läsionsmodell, wie der Quetschläsion, die die überwiegende Ursache für Querschnittslähmung darstellt, ebenfalls zur lokomotorischen Erholung führt. Des Weiteren sollte in zukünftigen Studien ein Vergleich von USSC und mesenchymalen Stammzellen in Betracht gezogen werden, da bislang unklar ist, welcher Stammzelltyp effizienter zur Behandlung traumatischer Rückenmarkverletzungen eingesetzt werden könnte.

2. Introduction

2.1 Stem cell characteristics

Stem cells are defined by their ability to self-renew and by their potential to differentiate into more mature progeny. In general, the stem cell differentiation potential is restricted to lineages that comprise the tissue of origin. Throughout lifespan, adult stem cells maintain tissue function by replacing differentiated cells when they get lost caused by attrition or damage. It has been demonstrated that a loss of stem cell self-renewal capacity leads to certain degenerative diseases and is probably one component of the aging process (Rando, 2006; Janzen et al., 2006). Normally, self-renewal is properly controlled by the local tissue microenvironment that maintains and regulates normal homeostatic cell division and stem cell fate. These stem cell niches sequester stem cells from differentiation and apoptotic stimuli as well as uncontrolled cell division. This is of importance since it has been shown that a loss of cell fate control results in large numbers of descendent cells disrupting tissue architecture followed by tissue or organ failure (Reya et al., 2001).

Largely, adult stem cells are quiescent meaning resting in the G0 or G1 phase of the cell cycle. A stem cell can give rise to two identical daughter cells after symmetric cell division or two non-identical daughter cells after asymmetric cell division. Most stem cells undergo asymmetric cell division when they are in their natural environment (Knoblich, 2008). During asymmetric cell division, several proteins are unequal segregated to one of the two daughter cells (Betschinger et al., 2006). These proteins are defined as segregating determinants as they specify daughter cell fate (Fig. 2.1A). Regulators of self-renewal are localized asymmetrically during mitosis while they are inherited by only one of the two daughter cells (Betschinger and Knoblich, 2004; Yu et al., 2006). Another hypothesis is that the stem cell fate depends on direct contact to the niche surface (Fig 2.1B) as the mitotic spindle is perpendicularly oriented which ensures that only one daughter cell can maintain contact with the stem cell niche and retain the ability to self-renew (Yamashita et al., 2003; Knoblich, 2008).

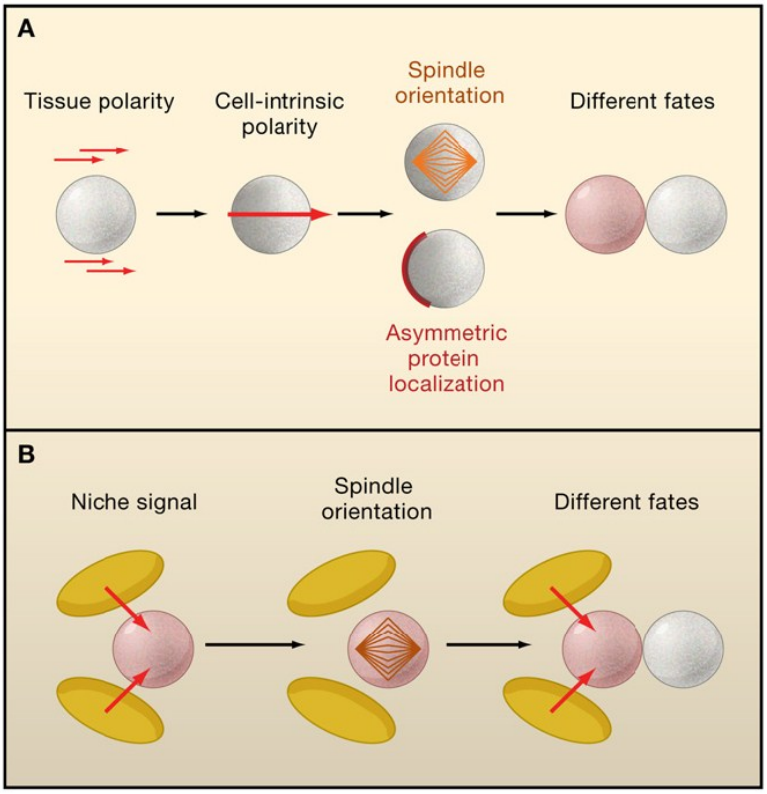

Fig. 2.1 Regulation of asymmetric stem cell division. Asymmetric cell division gives rise to two cells with different cell fates. The stem cell fate depends on asymmetric segregation of cell fate determinants during mitosis (A) or on signals from the niche surface which retain the ability of one daughter cell to self-renew (B). The orientiation of the mitotic spindle along the same polarity axis ensures the asymmetric segregation (A) whereas orientation of the mitotic spindle peripendicular to the niche surface (B) ensures that only one daughter cell continues to receive signals to maintain the ability to self-renew (according to Knoblich 2008).

Asymmetric cell division gives rise to two cells which have different cell fates. One daughter cell remains as a stem cell in the niche while the other is committed to a lineage specific differentiation. The precursor cell leaves the stem cell niche migrating to its target location where it differentiates and contributes to tissue function, as shown e.g. for the subventricular zone (SVZ) in the adult brain. The SVZ stem cell niche generates neuroblasts which migrate along the rostral migratory stream (RMS) to the olfactory bulb (Lois et al., 1996) where they differentiate into

GABAergic neurons that integrate into the granule cell layer contributing to olfactory learning (Magavi et al., 2005).

2.2 Stem cell types

Numerous terms exist to describe different stem cell types which are not always been used in a consistent way. Stem cells can be considered as embryonic stem cells (ESC) or as adult stem cells which are found in mature tissues of different organs.

Each stem cell has a specific ability to differentiate into numerous cell types dependent on its potency. Only zygotes are considered to be totipotent cells, since they are the only cells which give rise to both every celltype of the embryo and the trophoblast of the placenta. This ability distinguishes them from pluripotent stem cells, which are able to differentiate into all cell types of the embryo except the trophoblast. These cells are usually referred to as embryonic stem cells. In contrast, the differentiation potential of multipotent stem cells which are usually adult stem cells is limited to a subset of celltypes.

Another accepted nomenclature of different stem cell types is related to their tissue specificity. For example, adult stem cells of the nervous system are known as neural stem cells (NSC) whereas blood-forming haematopoietic stem cells (HSC) circulate in the blood.

The potential of stem cells to generate various cell types has emerged great interest for pre-clinical and clinical investigations to cure and repair injured tissues. In the last decade, extensive progress has been made on characterization, isolation, *in vitro* differentiation and transplantation of multiple stem cell types into various pre-clinical animal disease models. Replacement of lost endogenous cells either by transplantation or by recruitment of resident stem cells is the main goal of stem cell research. Investigations focus on cell replacement strategies for degenerative disorders such as Alzheimer´s or Parkinson´s disease, to cure diabetes with pancreatic cells differentiated from stem cells, to replace damaged cells after myocardial infarction or to replenish immune deficiencies. It is further suggested that stem cells could offer several other approaches for tissue repair as they might

facilitate regeneration by providing trophic factors or a permissive substrate for regeneration of endogenous cells.

2.2.1 Embryonic stem cells

ESC are pluripotent cells derived from the inner cell mass from mammalian blastocyts or from germ cells which can be differentiated into clinically relevant cell types of ectoderm, mesoderm and endoderm *in vitro*. Further, ESC can be cultured under defined conditions on a large-scale while they maintain their broad differentiation capacity. These features appear to be ideal for clinical cell replacement approaches and regenerative medicine. Unfortunately, teratocarcinoma formation which was observed after transplantation of ESC in different mouse models limits the clinical translation. Tumour formation is supposed to arise by direct transformation of the transplanted ESC followed by uncontrolled proliferation (Erdö et al., 2003). It is currently believed that a pre-differentiation into defined phenotypes and a purification of progenitor cells would probably obviate teratoma formation. A public debate has emerged focussing on the ethical problems associated with the destruction of the embryo during harvesting of ESC. Therefore, alternative methods of establishing pluripotent stem cells that do not interfere with the developmental potential of embryos are studied extensively. Interestingly in this regard, ESC can be established without any reduction of the developmental capacity of the embryo with a single cell embryo biopsy which could possibly circumvent the ethical concerns (Chung et al., 2006).

2.2.2 Adult stem cells

In some organs, such as the bone marrow, hair follicle, epidermis, gut and brain, stem cells reside throughout lifetime. In other organs, stem cells divide only after stimulation, usually in response to stress. Haematopoietic stem cells (HSC) are the best characterized adult stem cells (Weissman, 2000; Kondo et al., 2003). HSC reside in the adult bone marrow which further contains different haematopoietic and

non-haematopoietic cells. Osteoblasts (mesenchymal cells producing bone matrix), which are localized close to the bone, are known to form and maintain the function of the HSC niche (Deguchi et al, 1999; Ducy et al., 2000; Calvi et al., 2003). HSC can be directly isolated from bone marrow but also after peripheral blood mobilization and from human umbilical cord blood which are successfully used for reconstitution of the haematopoietic system after bone marrow ablation.

In addition to HSC, another stem cell population resides in the bone marrow, named mesenchymal stem cells (MSC). These cells give rise to bone, cartilage, adipose and fibrous tissue (Pittenger et al., 1999). It has been suggested that MSC are in close association with HSC to retain their quiescence (Benvenuto et al., 2007) and maintenance (Méndez-Ferrer et al., 2010). However, purification of a homogenous MSC population from bone marrow is lacking due to an uncompleted cell marker profile. In contrast, crypt base columnar cells were recently characterized as gut epithelial stem cells by an exclusive expression of Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as Gpr49). These cells self-renew and give rise to differentiated cells that constantly repopulate the villi (Barker et al., 2007).

Another stem cell source is the bulge of a hair follicle where multipotent stem cells differentiate to all epithelial cells within the hair follicle (Morris et al., 2004) and contribute to wound repair in the epidermis (Ito et al., 2005). Interestingly, transplanted hair follicle stem cells are able to generate hair follicles, hair and skin epidermis (Claudinot et al., 2005). These findings provide potential for the treatment of hair loss and other disorders of hair and skin.

The existence of neural stem cells in the adult mammalian brain is well established for two different regions: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. In the SVZ, slowly dividing, radial glia-like cells have been identified as primary neural stem cells. Once activated, these cells generate rapidly dividing transient amplifying precursor cells which subsequently give rise to neuroblasts that migrate to the olfactory bulb (OB) (Lois and Alvarez-Buylla, 1994; Lois et al., 1996). In the OB, neuroblasts primarily differentiate into GABAergic and dopaminergic interneurons. NSC are also found in the SGZ generating actively self-renewing progenitors and neuroblasts which mainly

differentiate into local neurons. Although NSC have been isolated from other brain regions, active neurogenesis seems to be restricted to SVZ and SGZ in the adult mammalian brain. NSC are the most promising stem cells for clinical applications in neurodegenerative diseases as they can easily give rise to mature neurons and oligodendrocytes. First initial clinical trials to replace lost cells by NSC or their differentiated progeny were performed with patients affected by different neurological diseases. However, one patient developed several neuroblastoma that have been derived from the graft (Amariglio et al., 2009), showing that safety and tumorigenicity of NSC transplantation has to be reconsidered.

2.2.3 Stem cells derived from human umbilical cord blood

Haematological malignancies often require the transplantation of haematopoietic stem cells which can be isolated from bone marrow, from blood after peripheral blood mobilization or from human umbilical cord blood (hUCB). Cord blood was mainly used for screening procedures of blood parameters of the newborn until the first successful cord blood transplantation in 1989 (Gluckman et al., 1989). Up to now, cord blood has emerged as an accepted alternative source of HSC for transplantation in patients. As suitable HLA (human leukocyte antigen) matched related donors are unavailable for many patients, cord blood from unrelated donors have been increasingly used as an alternative stem cell source for adult patients (Arcese et al., 2006; Lekakis et al., 2006; van Heeckeren et al., 2007). A lower risk for acute and chronic graft-versus-host-disease (GVHD) despite major HLA disparity compared to bone marrow transplants, the immediate availability and the risk-free donation are further advantages of umbilical cord blood. Unfortunately, it is difficult to receive a single cord blood unit of satisfactory nucleated cell dose for adult patients. In order to overcome that limited number of cells, double cord blood transplantation from different donors has been recognized as one of the most attractive strategies (Barker et al., 2005). Another approach to get a sufficient cell number for transplantation is the *ex vivo* expansion of cord blood cells (Pecora et al., 2000; de Lima et al., 2008).

In addition to HSC, mesenchymal stem cells (MSC) can be isolated from umbilical cord blood and expanded as adherent, fibroblastic-like cells *in vitro*, which feature characteristics similar to MSC populations from bone marrow. MSC are defined as multipotent cells which can be differentiated into diverse cell types, e.g. osteocytes, chondrocytes and adipocytes (Goodwin et al., 2001; Gang et al., 2004). On the other hand, ectodermal and endodermal differentiation potential of MSC from both bone marrow and umbilical cord blood is controversially discussed since different *in vitro* differentiation protocols have been described to induce marker expression but *in vivo* confirmation is still lacking. Instead of cell replacement, MSC are thought to provide trophic support which might influence the recruitment of endogenous stem cells to the injured tissue. Moreover, MSC are known to have immunomodulatory properties as they can inhibit T-cell proliferation *in vitro* and *in vivo* (Zappia et al., 2005; Gerdoni et al., 2007). In animal models of clinical relevant diseases, MSC transplantation was shown to have beneficial effects. Interestingly, it has been demonstrated that MSC can decrease oligodendrocyte apoptosis, demyelination and clinical signs after transplantation into an animal model of multiple sclerosis (Zhang et al., 2009). In a model of amyotrophic lateral sclerosis (ALS), MSC transplantation has been shown to reduce astrogliosis and microglial activation while increasing motor neuron survival and motor performance (Vercelli et al., 2008). Rather little is known about the involved mechanisms but the promising results make MSC attractive for further investigations.

2.2.4 Mesenchymal stem cells compared to unrestricted somatic stem cells

MSC from human umbilical cord blood (CB-MSC) were first described by Erices and colleagues (Erices et al., 2000), which revealed an immunophenotype similar to that of bone marrow. In 2004, Kögler and colleagues (2004) detected another stem cell type in the cord blood with an even broader differentiation potential than CB-MSC, named unrestricted somatic stem cells (USSC). USSC and CB-MSC cultures can be generated by the same method and cultured as spindle-shaped adherent cells.

Although USSC have several features in common with human MSC from bone marrow and cord blood, like e.g. the osteogenic and chondrogenic *in vitro* and *in vivo* differentiation potential, they clearly vary in their immunological behavior (van den Berk, 2009; Winter et al., 2009) and their transcriptome (Jansen et al., 2010). It has been demonstrated that USSC can be well characterized by the expression of DLK-1/PREF-1 (delta-like 1/preadipocyte factor-1) that inhibits adipogenic differentiation whereas CB-MSC have the intrinsic potential to give rise to mature adipocytes (Kluth et al., 2010). In addtion, USSC can be distinguished from CB-MSC by a specific Hox gene expression pattern (negative Hox-code; Liedtke et al., 2010). Moreover, USSC possess longer telomeres, repeated DNA sequences at the ends of chromosomes - the shortening of which is linked to a decrease in the ability of cells to divide, compared to CB-MSC and MSC from bone marrow. Further, with only 30-45% USSC exhibit a lower senescence rate, whereas already 70-80% of CB-MSC are considered senescent after nine passages in culture. In comparison, almost 95% of MSC from bone marrow are senescent after nine passages (Aktas et al., 2010). Importantly, a tumorigenic potential of USSC could be excluded as revealed in a 16 month study in which xenograft tumorigenicity was tested in immunodeficient mice (Aktas et al., 2010). Hence, USSC are likely to be suitable for transplantation studies. Favorably, USSC can be purified in a GMP-grade status (Aktas et al., 2010) without any ethical concerns and invasive interventions. Moreover, USSC are readily available as they can be easily expanded in a clinical scale. Our group demonstrated that USSC differentiate *in vitro* into neuronal-like cells after a specific induction (XXL-medium incubation) characterized by cell cycle exit (Greschat et al., 2008; Trompeter et al., 2010) and the expression of various neuronal markers such as neurofilament, β-III-tubulin, synaptophysin, and NeuN (Greschat et al., 2008). Although the microRNA profile indicates a differentiation into immature neural cells (Iwaniuk et al., 2010), further maturation into functional, action-potential competent neurons failed after XXL-induction, co-cultivation with primary neural cell cultures and organotypic slices. On the other hand, high expression of various interleukins (e.g. IL-1, IL-6) and growth factors like stromal cell-derived factor-1 (SDF-1), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Kögler et al., 2005) are

characteristics of USSC. To this end, USSC are an attractive human stem cell population for clinical applications in CNS injuries.

2.3 Stem cell migration

Most stem cell types and their progenitors show remarkable cell motility. Chemoattractants and cell-surface adhesion molecules, including selectins and integrins, are crucial for cell migration. It was demonstrated that the majority of haematopoietic stem cells (HSC) can exit the endosteal bone marrow HSC niche, known as mobilization occurring during homeostasis (Wright et al., 2001). The chemokine CXCL-12, also known as stromal cell-derived factor-1 (SDF-1), has been identified as a key factor involved in migration and mobilization of HSC during homeostasis (Hattori et al., 2001; Ara et al., 2003). Furthermore, HSC migration depends on the interaction of membrane bound stem cell factor (SCF) expressed by osteoblasts and the stem cell factor receptor KIT which itself is highly expressed by HSC. Mutations disturbing these interactions affect migration and differentiation of HSC (Lyman and Jacobsen, 1998). Interestingly, the migration of neural crest cells, which generate multiple components of the peripheral nervous system (PNS), is modulated by SDF-1 (Kasemeier-Kulesa et al., 2010). In the CNS, CXCR-4 receptor expression and a migration along SDF-1 gradients have been described for granule neuron precursors (Zou et al., 1998; Ma et al., 1998) and for developing cortical interneurons (Stumm et al., 2003). On the other hand, migration of neuroblasts from the SVZ to the olfactory bulb in the adult mammalian brain depends on sonic hedgehog (Shh) and insulin-like growth factor-1 (IGF-1) (Balordi and Fishell, 2007; Hurtado-Chong et al., 2009). Additionally, migration along the RMS is regulated by interactions between neuroblasts and the local RMS microenvironment (Garcia-Marques et al., 2010).

It has been proposed that circulating or resident stem cells as well as transplanted stem cells are selectively recruited to a site of injury. After transplantation into recipients with bone marrow ablation, HSC have the capacity to provide lifelong reconstitution of all blood-cell lineages, even using a single cell

(Osawa et al., 1996; Yang et al., 2005). HSC are mostly given intravenously after chemotherapy and follow a gradient of SDF-1 which subsequently directs them to the bone marrow. Interestingly, recent reports revealed that SDF-1 is upregulated by astrocytes and endothelial cells (Rubin et al., 2003; Rezaie et al., 2002) after ischemic brain lesion as well as after myocardial infarction consequently attracting transplanted bone marrow stromal cells (Hill et al., 2004; Wang et al., 2008; Yu et al., 2010) and mononuclear cells from human umbilical cord blood (Rosenkranz et al., 2010). Further, it was shown that endogenous or transplanted neural stem cells (NSC) respond to SDF-1 which directs their migration in a chain-like fashion to the source, allowing NSC to home to the pathology (Imitola et al., 2004; Robin et al., 2006; Itoh et al., 2009).

Another key factor involved in cell migration is the hepatocyte growth factor (HGF), also known as scatter factor, which has even described as a chemoattractant for various stem cell types (Neuss et al., 2004; Son et al., 2006). HGF is a 90 kD multidomain glycoprotein which binds to its receptor c-met. It is secreted as a single-chain inactive polypeptide and cleaved to its active α/β heterodimer by proteases (Birchmeier et al., 2003). HGF binding induces receptor homodimerization and autophosphorylation activating multiple downstream signals. A wide range of effectors have been identified involved in cell motility induced by HGF (Fig. 2.2). Activation of PI3K, Akt, ERK/MAPK and Rac1 have been proposed to modulate HGF dependent cell motility (Khwaja et al., 1998; Kodama et al., 2000; Delehedde et al., 2001, Coltella et al., 2003). Recent reports revealed that HGF signalling plays a key role during migration of interneurons through forebrain development (Powell et al., 2001), spinal motor neuron attraction (Ebens et al., 1996) and myoblast migration (Bladt et al., 1995).

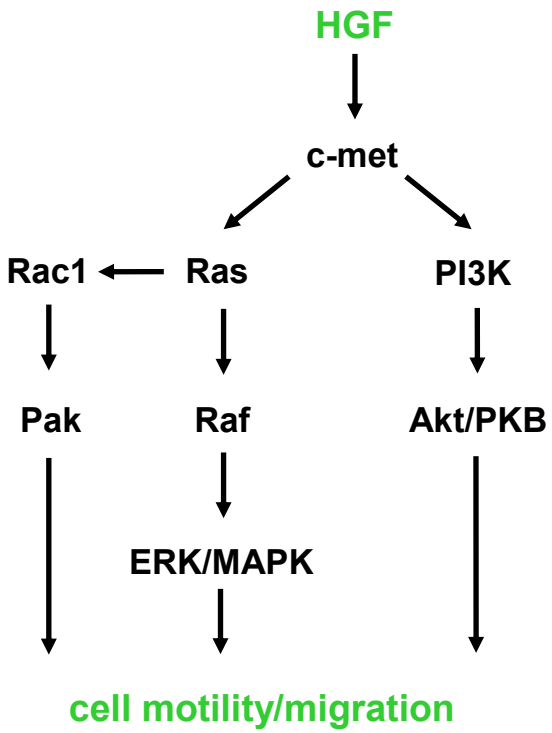

Fig. 2.2 HGF and its downstream signaling pathways. After dimerization and autophosphorylation of the HGF receptor c-met various downstream signals are activated, e.g. Rac1, ERK/MAPK and Akt/PKB which modulate HGF-dependent cell motility (according to Segarra et al., 2006, with modifications).

Of note, it was demonstrated that HGF is upregulated due to tissue damage, as shown for ischemic brain injury (Honda et al., 1995) and myocardial ischemia (Miyazawa et al., 1994) which is suggested to attract transplanted mesenchymal stem cells (Neuss et al., 2004; Trapp et al., 2008; Vogel et al., 2010).

2.4 Stem cells applied in the present study

In the present study, two different USSC populations (SA 5/03 and SA5/73) were used which were provided by the group of Prof. Dr. Gesine Kögler from the Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Medical Center, Düsseldorf. This well-defined human stem cell population has been

used for the first time as a cellular therapeutic strategy in a pre-clinical animal model for spinal cord injury.

2.5 Molecular and cellular events after spinal cord injury

Injury of the spinal cord interrupts the electrophysiological signal transduction resulting in long lasting loss of mobility and sensory input as well as autonomic nervous system control below the level of the lesion. Injured spinal cord axons degenerate while compensatory recovery is limited. Worldwide, spinal cord injury (SCI) affects over 2.5 million people (2005, http://www.rickhansenregistry.org). High-energy trauma is the usual reason for SCI which is heterogeneous in cause and outcome (Kakulas 1999). Contusion as a result of fracture dislocation of the spine is the most common kind of injury. The survival rate of the individual is good, but the patient suffers from handicaps diminishing life quality generating personal, societal and economic costs (Rossignol et al., 2007). Currently, no curative therapy is available. Progressive expansion of the injury across more than one segment can occur over month or years, sometimes proving fatal (Thuret et al., 2006).

After traumatic spinal cord injury, spontaneous regeneration of CNS axons fails due to massive cell death, presence of extrinsic inhibitory molecules and the deficient growth potential of adult CNS neurons. Primary loss of cells at the site of the injury initiates various inflammatory responses as immune cell infiltration due to the interrupted blood spinal cord barrier. Additionally, acute central hemorrhagic necrosis activates glial cells and leukocyte infiltration (Fleming et al., 2006). Formation of a glial scar by reactive astrocytes and a fibrous scar in the lesion center composed of fibroblastic meningeal cells limit further damage by suppressing inflammation and preserve the function of the salvageable tissue (Faulkner et al., 2004). Although scar formation limits further tissue loss, it prevents axonal regeneration acting as a physical and a chemical barrier including various growth inhibitory molecules. Presence of chondroitin sulfate proteoglycans (CSPGs), keratin sulfate proteoglycans, tenascins, ephrins and semaphorins characterizes the extracellular matrix (ECM) which surrounds the lesion site and contributes to scar formation. This

barrier is thought to be a major factor limiting axonal regeneration after CNS injury (Stichel and Müller, 1998; Fawcett and Asher, 1999; Grimpe and Silver, 2002; Silver and Miller, 2004; Klapka and Müller, 2006). Additionally, myelin-associated molecules such as Nogo-A, MAG (myelin-associated glycoprotein) and OMgp (oligodendrocyte myelin glycoprotein) originating from myelin debris are enriched in the scar tissue and represent a further limitation of axonal regeneration (Caroni and Schwab, 1988; Wang et al., 2002; Mukhopadhyay et al., 1994; McKerracher et al., 1994). Upon contact with axons, these inhibitors induce intracellular events leading to a collapse of newly formed growth cones and to growth arrest (Filbin 2003, Schwab et al., 2005).

Damage of spinal cord tissue results in secondary degeneration which leads to cell death of multiple cell types, often triggered by lipid peroxidation, an increase of free radicals and excitotoxic transmitter levels (glutamate and aspartate). Loss of oligodendrocytes and a deficient expression of myelin-associated genes further impair the function of remaining axons. Unfortunately, chronically demyelinated axons are vulnerable for degeneration. Of note, in some cases limited spontaneous repair occurs after CNS injury. Thus, in order to find an effective and safe therapeutic strategy to cure SCI, further investigations about the cellular and molecular mechanisms are required to evaluate the failure of axon regeneration in the CNS.

2.6 Axonal de- and regeneration after spinal cord injury

Regeneration of injured nerve fiber tracts occurs spontaneously in the embryonic CNS as well as in the adult PNS while regrowth in the adult CNS is limited. Interestingly, transplantation studies with peripheral nerve grafts into the injured CNS led to an extensive CNS axon regeneration, indicating that CNS neurons have the intrinsic capability to regenerate (David and Aguayo, 1981, Benfey and Aguayo, 1982). In addition, limited sprouting of the proximal stump has been observed after CNS lesion which stops its growth at the lesion site (Beattie et al., 1997; Stichel and Müller, 1998). These experiments revealed that lack of axonal regeneration after

CNS trauma is not primarily due to an inherent lack of axonal growth potential, but rather the presence of axonal inhibitors in the CNS.

Soon after axotomy, the plasma membrane seals at the cut end resulting in axon retraction from the site of injury followed by the formation of a growth cone. Axonal regrowth to the target area and subsequent synaptogenesis depend on the neuronal cell type, age and distance of the injury site from the cell body. After peripheral nerve injury, damaged axons distal to the site of lesion undergo Wallerian degeneration (Becerra et al., 1995). Cell and myelin debris is removed immediately after injury, mainly by Schwann cells and macrophages leading to a permissive environment for regenerating axons. In contrast, debris clearance is much slower in the CNS (George and Griffin, 1994). Moreover, numerous regeneration-associated genes are upregulated, including the activating transcription factor-3 (ATF-3, Seijffers et al., 2006) and c-Jun (Raivich et al., 2004) after PNS injury, whereas injury of CNS axons does not result in activation of these genes. These results demonstrate that PNS axons have a superior ability to regenerate compared with those of the CNS (Afshari et al., 2009). The limited capacity of the CNS to react with an intrinsic pro-regenerative response may be due to the need for the complex CNS neuronal network to limit possible aberrant re-wiring (Di Giovanni, 2009). After injury of the PNS, Schwann cells which normally myelinate PNS axons, dedifferentiate, proliferate and align longitudinally after axotomy to form bands of Büngner guiding regenerating PNS axons (Chaudhry et al., 1992). In contrast, reactive astrocytes form a physical barrier including secreted and transmembrane bound growth inhibiting molecules blocking CNS axon regeneration. Due to demyelination of CNS axons after injury, myelin-associated inhibitors are released and accumulate in the glial scar. These inhibitory molecules could, with exception of MAG which is rapidly eliminated by macrophages, not be identified in the PNS.

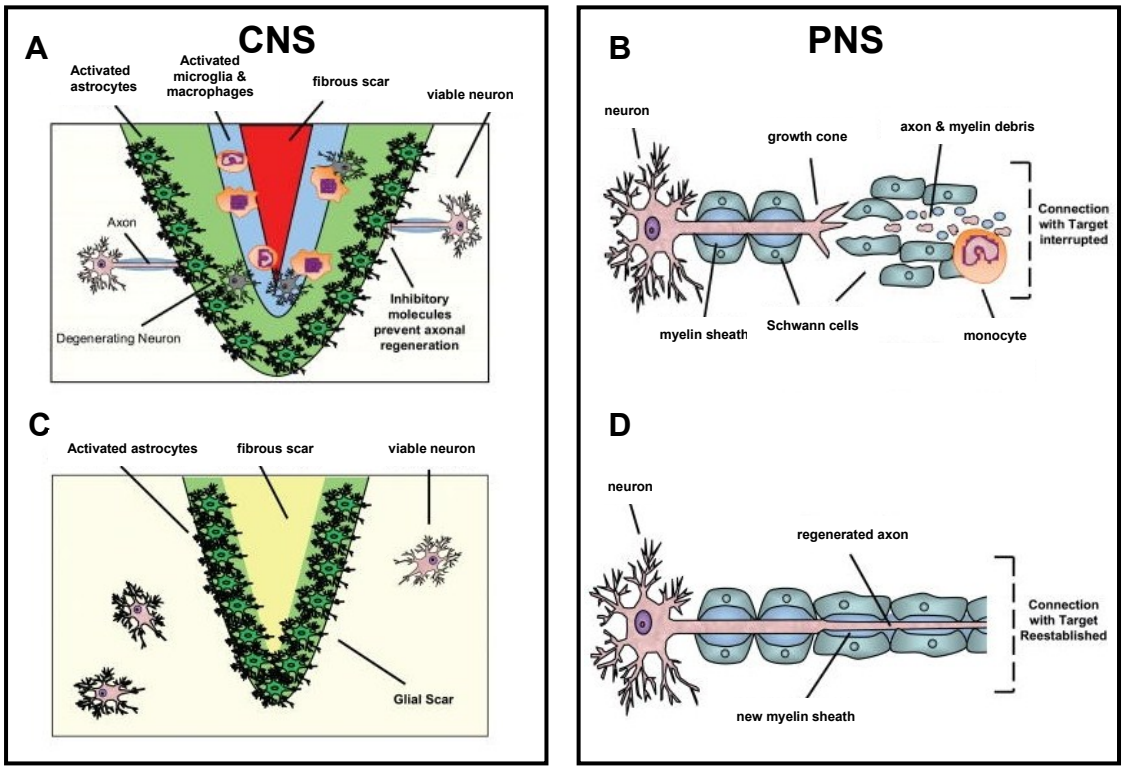

Fig. 2.3 Overview of CNS and PNS responses after injury (A) After CNS injury, microglia and macrophages are recruited to the lesion site, secreting various cytokines and growth factors, while removing necrotic tissue. The formation of a glial scar by reactive astrocytes and a fibrous scar by invading meningeal fibroblasts in the lesion center limits further damage by suppressing inflammation and preserve the function of the salvageable tissue. The presence of inhibitory molecules accumulating in the scar tissue inhibits regeneration of damaged axons. (B) Following PNS injury, monocytes and macrophages are recruited to the lesion and immediately phagocytose axon and myelin debris. Schwann cells dedifferentiate, proliferate and align longitudinally to form a scaffold (bands of Büngner) guiding regenerating PNS axons. (C) CNS injury leads to secondary degeneration which is often accompanied by a cerebral spinal fluid–filled cyst. The presence of collagenous tissue and inhibitory molecules which attach to the ECM of the fibrous scar are the main components for the failure of regeneration. (D) Axons successfully innervate their targets while Schwann cells produce new myelin to insulate the regenerated axons after PNS injury (according to Reichert, 2008, with modifications).

To achieve efficient axonal regeneration, different strategies were evaluated in various rodent SCI models, resulting in some degree of functional recovery, which will be described further below.

2.7 Current therapeutic approaches

SCI research mainly focuses on the improvement of axon regeneration, since regrowth of a low number of axons can lead to recovery of some respectable motor or sensory functions (Fawcett, 1998). Further, spinal plasticity, including sprouting of injured corticospinal axons, was shown to contribute to limited compensatory recovery due to an increased connectivity (Hill et al., 2001; Raineteau and Schwab, 2001; Weidner et al., 2001). Tissue protection from secondary cell death is another main intend of therapeutic interventions after SCI. For successful regeneration, axons must be able to initiate the process of regrowth followed by a dynamic process to reconnect to appropriate targets for synapse formation (Afshari et al., 2009) and thereby reconstitute original or equivalent circuitry which will lead to functional restoration. As introduced in 2.5, various mechanisms and molecules are identified as being inhibitory for axonal regeneration. Based on these observations, different cellular and molecular therapeutic strategies have been described to achieve, improve and direct axonal regeneration in addition to locomotor training. Currently, none of the reported interventions has fulfilled all the criteria for efficient restoration (Bradbury and McMahon, 2006).

2.7.1 Molecular therapeutic approaches

Molecular therapeutic interventions after SCI focus on axon regeneration by application of growth promoting molecules, blocking of inhibitory molecules, modifications of the scar tissue and limitation of secondary damage.

Expression of neurotrophic factors is abundant in the developing CNS, but decreases in the adult. Exogenous delivery of neurotrophic factors, which could modulate neuronal survival and axonal regeneration, as well as the manipulation of pro-regenerative neuronal signaling pathways has been proposed as therapeutic strategies for SCI. Application of various growth factors has been tested in pre-clinical studies, e.g. brain-derived neurotrophic factor (BDNF, McTigue et al., 1998; Jakeman et al., 1998), fibroblast growth factor (FGF, Lee et al., 1999), glial-derived neurotrophic factor (GDNF, Brock et al., 2010), nerve growth factor (NGF, Grill et al.,

1997), and neurotrophin-3 (NT-3, Taylor et al., 2006). Indeed, BDNF was shown to induce locomotor function recovery and axonal growth of rubrospinal and raphespinal axons as well as local sprouting of sensory and motor axons (Liu et al., 1999). Furthermore, delivery of GDNF enhances growth of sensory axons and contributes to remyelination (Blesch and Tuszynski, 2003). Moreover, it has been demonstrated that NGF induces growth of coerulospinal axons (Tuszynski et al., 1996), whereas NT-3 promotes growth of corticospinal axons (Grill et al., 1997). Among different signaling pathways engaged by neurotrophic factors, two major pathways have been most intensively studied involved in the regulation of axon growth: the PI3K/AKT pathway and the Ras-activated Raf/ERK kinase cascade (Park et al., 2010). It has been demonstrated that inhibition of either Ras or PI3K partially reduces axon outgrowth stimulated by different growth factors. Furthermore, Ras, Raf, MEK, PI3K and AKT are required for NGF-induced axon elongation (Fig. 2.4) (Markus et al., 2002; Park et al., 2004; Park et al., 2008). Of note, these signalling molecules are also known to mediate neuroprotection.

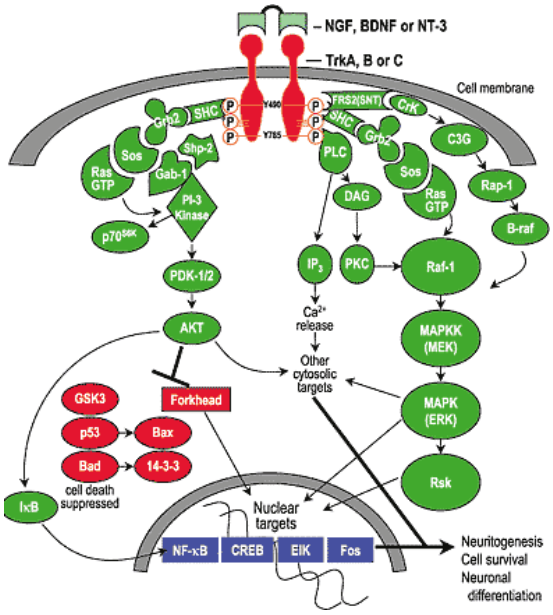

Fig. 2.4 Binding of neurotrophic factors to the corresponding receptors leads to axonal growth and cell survival. After activation of the receptors TrkA, B or C by neurotrophic factors (e.g. NGF, BDNF or NT-3), downstream signaling cascades result in enhanced neuritogenesis and cell survival. Among other pathways involved in axon growth and cell survival, the PI3K/AKT and the Ras-activated Raf/ERK kinase cascade are the most extensively studied pathways (according to Pollack and Harper, 2002).

In addition to the traditional neurotrophins, other regeneration- and neuroprotection-associated factors have been identified; such as the hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and the chemokine leukaemia inhibitory factor (LIF). It has been described that HGF promotes motoneuron survival and axonal regrowth (Miyazawa et al., 1998) and is further considered as a guidance and survival factor during neural development (Ebens et al., 1996; Giacobini et al., 2007). Experimental evidence implies that HGF is able to enhance regeneration due to promotion of neuronal and oligodendroglial survival, angiogenesis and axonal regeneration after SCI (Kitamura et al., 2007). Application of VEGF has been shown to support neuronal survival (Jin et al., 2000; Sun et al., 2003; Tovar-y-Romo and Tapia, 2010), neurite outgrowth (Khaibullina et al., 2004)

and tissue sparing in SCI (Kim et al., 2009). Furthermore, administration of LIF in a rodent model of acute SCI was shown to reduce oligodendrocyte apoptosis with a substantial decrease of demyelination (Kerr and Patterson, 2005; Azari et al., 2006). HGF and VEGF exert their effects through activation of PI3-K/Akt and the MEK/ERK signaling pathways (Shiote et al. 2005; He et al., 2007; Ma et al., 2007; Tolosa et al., 2008). In contrast, the major downstream signaling pathway for LIF is the Jak/STAT pathway (Janus kinase (Jak) / signal transducers and activators of transcription (STAT)) (Rajan et al., 1995; Taga 1996; Leibinger et al., 2009).

Strategies that intend to overcome inhibitory cues of the glial scar have been shown to result in significant axon regeneration through and beyond the injury site. Neutralization of Nogo-A by specific antibodies leads to long distance regeneration of descending axons, compensatory fiber growth and recovery of limb function (Merkler et al., 2001; Schwab, 2004; Liebscher et al., 2005; Mullner et al., 2008). However, evidence from Nogo-A knockout mouse studies remains controversial as the results vary with age, strain background, genetic mutation and type of lesion (Zheng et al., 2003; Simonen et al., 2003; Dimou et al., 2006; Lee et al., 2010). Degradation of CSPGs, which are known to inhibit neurite outgrowth *in vitro* and *in vivo* (Dou and Levine, 1994; Levine, 1994), by the bacterial enzyme chondroitinase ABC (ChABC) promotes axon regeneration and functional recovery (Moon et al., 2001; Bradbury et al., 2002). In addition, suppression of collagen synthesis resulting in modifications of the fibrous scar has led to a scar tissue which was more permissive for regenerating axons and promoted functional recovery after acute SCI (Klapka et al., 2005).

These individual treatments described above have proven efficacious in facilitating regeneration and functional recovery after SCI. However, the number of regenerating axons is generally low and the functional recovery modest. Therefore, combination of strategies could further enhance the beneficial effects. Several studies have demonstrated additive potential of two or more therapeutic approaches. For example, cell transplantations could be combined with neuroprotective agents (Pearse et al., 2004), exogenous or cellular overexpressed growth factors (Coumans et al., 2001; Sasaki et al., 2009) or ChABC (Chau et al., 2004; Fouad et al., 2005).

2.7.2 Cellular therapeutic approaches

The potential of stem cells to generate neural cell types has emerged great interest for SCI research. Numerous pre-clinical studies have indicated that transplantation of different stem cell types provide beneficial effects on regeneration after SCI. As damage to the spinal cord is accompanied by a severe loss of cells, cell replacement has been the primary intend for cell transplantation. After SCI, some axons still remain but demyelinate and consequently become non-functional. This observation suggests that replacement of degenerating myelinating oligodendrocytes by transplantation of stem cells or oligodendrocyte precursors might be beneficial.

On the other hand, beneficial effects have been demonstrated without any lineage-specific differentiation or obvious cell replacement. The underlying mechanism appears to be indirect, as transplanted cells might release growth promoting factors, modulate the inflammatory response, protect the endogenous tissue or provide a permissive environment for regenerating axons. For transplantation into the injured spinal cord, stem cells have been collected from different sources, among them embryonic stem cells isolated from the inner cell mass, fetal neural stem cells isolated from the brain and adult stem cells isolated from bone marrow (Fig. 2.5). Stem cells have been either propagated and manipulated *in vitro* before transplantation or directly transplanted into the injured spinal cord. Additionally, some celltypes have been used for autologous transplantation, including cells from the olfactory bulb or bone marrow therewith circumventing immune rejection and ethical considerations associated with the use of ESC or NSC. Unfortunately, various stem cell sources, transplantation methodologies, strain backgrounds, injury paradigms and functional behavioral tests make it impractical to compare the results and conclusions between the studies (Enzmann et al., 2006). Moreover, safeness and efficiency have been poorly demonstrated (Thuret et al., 2006) and only some studies report a long lasting effect beyond three month. Nevertheless, transplantation strategies are moving towards clinical trials. For example, recently oligodendrocyte precursors differentiated from human embryonic stem cells have been transplanted in a first patient to test safety and tolerability (http://www.geron.com).

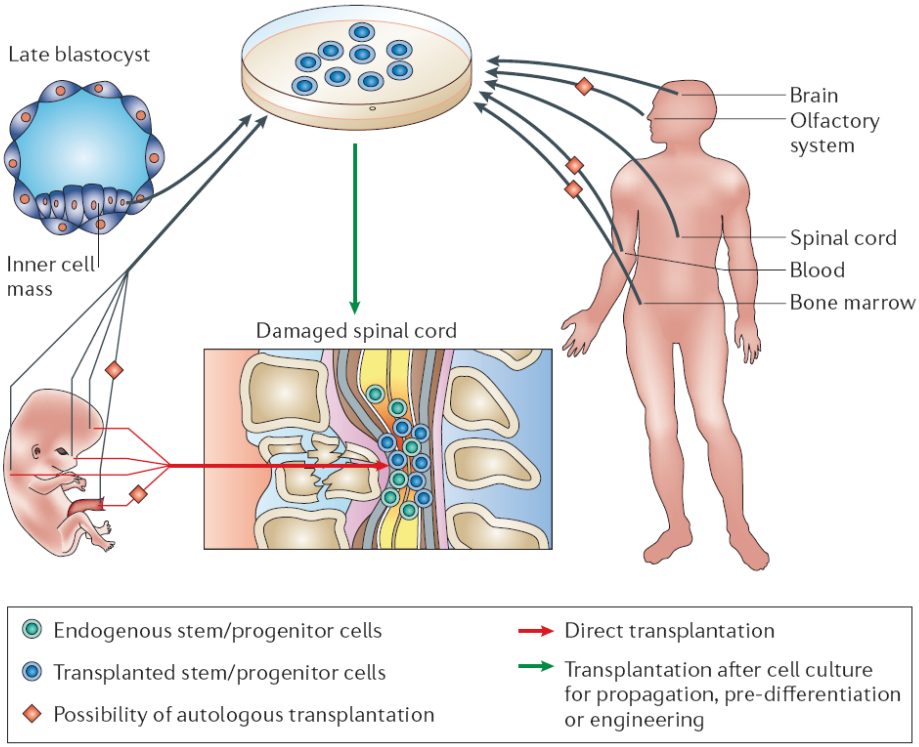

Fig. 2.5: Transplantation of stem cells from different sources into the injured spinal cord. Embryonic, fetal or adult stem cells can be either propagated and manipulated *in vitro* before transplantation or directly transplanted into the injured spinal cord. Furthermore, cells from the brain, olfactory bulb, umbilical cord blood, spinal cord or bone marrow can be used for autologous transplantation (according to Thuret et al., 2006).

2.7.2.1 Transplantation of embryonic and neural stem cells

NSC isolated from fetal or adult tissue or differentiated from ESC have been transplanted in a range of pre-clinical animal models (see review Tetzlaff et al., 2010). It has been reported that NSC transplanted into the injured spinal cord predominantly differentiate into astrocytes (Cao et al., 2001, Cao et al., 2002), but also an expression of oligodendroglial markers could be shown (Parr et al., 2007; Parr et al., 2008; Karimi-Abdolrazaee et al., 2006). However, neuronal differentiation of transplanted NSC was rarely observed. It has been suggested that NSC transplanted into the injured spinal cord lack neuronal differentiation due to inhibitory

signals and less survival, growth and guidance factors (Cao et al., 2002). Importantly, timing of transplantation, the host microenvironment, the phenotype and purity of the transplanted cells critically influence the efficiency of neuronal replacement (Rossi et al., 2009). Most of the NSC transplantation studies revealed significant improvement in open field locomotion (BBB) (Karimi-Abdolrezaee et al., 2006; Parr et al., 2008; Hofstetter et al., 2005). Interestingly, lowering of sensory thresholds to non-noxious stimuli (i.e. allodynia) was reported. Consequently, NSC could have the potential to promote neuropathic pain (Hofstetter et al., 2005). Additionally, the use of NSC harvested from human fetal tissue or human embryonic stem cells reveals ethical and technical difficulties, implying teratoma formation due to transplantation of a neural precursor cell population including a few undifferentiated ESC.

2.7.2.2 Transplantation of mesenchymal stem cells

Human adult MSC derived from bone marrow or peripheral blood stem cell mobilization have been transplanted in SCI animal models and in pilot clinical studies (Himes et al., 2006; Chernykh et al., 2007; Deda et al., 2008; Geffner et al., 2008; Pal et al., 2009, Cristante et al., 2009). These reports showed that transplantation of human MSC is without any risk for the patients in regard to allergic or inflammatory reactions and tumour formation (Geffner et al., 2008). Potential improvement of sensory and motor activity was reported in some studies (Himes et al., 2006; Chernykh et al., 2007; Cristante et al., 2009) which have to be critically checked and confirmed in independent clinical studies. A combined therapy of MSC transplantation and molecular therapeutics was suggested as a possibility to enhance the beneficial effect (Furuya et al., 2009; Luo et al., 2009)

Pre-clinical studies with human MSC in rodent SCI models showed variable mechanisms for measurable effects. Cizkova et al. (2006) reported that transplanted human MSC express oligodendroglial and neuronal proteins after transplantation into a compression injury, whereas Sheth et al. (2008) could not detect differentiation of transplanted cells into the neural lineage. The possibility of MSC to transdifferentiate into neural cells remains to be investigated (Phinney and Prockop, 2007). Despite the

lack of transdifferentiation, Hofstetter et al. (2002) reported that MSC from rat could longitudinally arrange and bridge the epicentre of the SCI which led to functional recovery. Others take into account that less cavity formation and an increased regeneration is due to their potential to secrete trophic factors which could be neuroprotective and regeneration-associated (Wu et al., 2003; Ankeny et al., 2004; Cizkova et al., 2006). Additionally, some reports indicated moderate functional improvement after human MSC grafting tested by open field locomotor scoring (Himes et al., 2006, Cizkova et al., 2006), whereas other groups could not assess functional improvement (Neuhuber et al., 2005; Sheth et al., 2008).

Most studies used human MSC separated from the haematopoietic cell fraction by their ability to adhere to plastic. Consequently, the transplanted cell population contained a mixture of stromal cells and MSC. Thus it is unclear which cell population was effective. In spite of available markers to characterize human MSC, the actual stromal *versus* MSC nature of transplanted cells remained unclear in most studies (Tetzlaff et al., 2010).

With a few exceptions, human MSC transplantation is typically performed subacutely (1-2 weeks after injury), since transplantations performed immediately after injury generally yield poor results due to the robust inflammatory response initiated at the time of injury. Moreover, transplantation of MSC into a chronic SCI model seems to lack functional recovery (Lu et al., 2007).

2.7.2.3 Transplantation of umbilical cord blood stem cells

In recent studies, human MSC isolated from umbilical cord blood have been transplanted into subacute lesions of moderate contusion spinal cord injury (Dasari, 2007; Dasari, 2008). It was demonstrated that CB-MSC could have a beneficial influence on tissue regeneration due to a downregulation of pro-apoptotic genes (Dasari et al., 2009) and release of trophic factors (Dasari et al., 2007). Further, Dasari et al. (2007) reported differentiation of transplanted CB-MSC towards the oligodendroglial lineage and remyelination of endogenous axons. Functional studies revealed a moderate locomotor improvement immediately after CB-MSC

transplantation into SCI (Dasari et al., 2007; Cho et al., 2008) and in other neurodegenerative disease models like stroke (Pimentel-Coelho et al., 2010) and Alzheimer´s disease (Lee et al., 2010). In the present study, USSC derived from hUCB have been used for the first time as a cellular therapeutic strategy in a pre-clinical animal model for spinal cord injury.

2.8 Aim of this thesis

Injury of the spinal cord results in a long lasting loss of mobility and sensory input as well as impaired autonomic nervous system control below the level of the lesion. Spontaneous regeneration of CNS axons fails due to scar formation, presence of extrinsic inhibitory molecules and the deficient growth potential of adult CNS neurons. Numerous pre-clinical studies have indicated that transplantation of different stem cell types provides beneficial effects on regeneration after SCI. However, transplantation studies with human derived stem cells isolated without any ethical concerns and invasive interventions are rare.

Although cord blood has emerged as an alternative source for somatic stem cells, it is still an open question whether stem cells from hUCB are therapeutically effective after transplantation into the injured spinal cord since thus far the results are inconsistent and the number of studies is insufficient. As stem cells derived from hUCB grafted into models of SCI mostly included barely defined heterogeneous MSC populations, transplantation studies of a well-defined stem cell population derived from hUCB is most likely needed. In addition to MSC, hUCB contains unrestricted somatic stem cells (USSC), which can be clearly discriminated from CB-MSC. Until now, USSC have not been used as a cellular therapeutic strategy in pre-clinical animal studies treating SCI. The investigative approach intended to answer

1. how long USSC survive after transplantation into a rat model of acute traumatic SCI,
2. whether USSC reveal migratory potential after transplantation,

3. whether USSC have a neural differentiation potential and will replace endogenous cells *in vivo*,
4. whether USSC transplantation leads to long-lasting functional locomotor recovery and
5. whether USSC transplantation enhances regenerative axon growth and prevents tissue loss.

3. Materials and Methods

3.1 Animals

Adult female Wistar rats weighing 180-210 g were used which were bred within the animal facility (Tierversuchsanlage, TVA) of the Heinrich Heine University, Düsseldorf. During the experiments, animals were housed under standard conditions (21°C, 50 ± 5% air humidity) with a 12h light/12h dark cycle. They were kept in groups in standard cages. Germ-free water and palletized dry food were available *ad libitum*.

All surgical interventions and pre- and post-surgical animal care were performed in compliance with the German Animal Protection law (State Office, Environmental and Consumer Protection of North Rhine-Westphalia, LANUV NRW; Az: 8.87-50.10.34.08.315). The numbers of rats used in each experimental group are listed in table 3.1.

Table 3.1: Experimental groups.

				animal groups		
survival time	Analysis	Lesion	Tracing	USSC	control	Sham
1d	*In vitro* USSC migration (tissue for under-agarose chemotaxis assay)	+ and -	no	no	4	4
1w	*In vivo* USSC localization/differentiation/migration	+ and -	no	5	5	5
3w	*In vivo* USSC localization/differentiation/migration	+	no	4	4	no
5w	*In vivo* axon regeneration into the lesion area	+	rostral	5	5	no
16w	*In vivo* functional recovery/tissue sparing	+ and -	no	14	9	7

3.2 Buffers/solutions and antibodies

3.2.1 Buffers/solutions

Table 3.2: Buffers and Solutions

Buffers/solutions	Supplier/Composition
0.2 M PB (phosphate buffer), pH=7.4	28.8 g Na_2HPO_4 5.2 g NaH_2PO_4 *ad* 1000 ml *aq. bidest*
0.1 M PBS (phosphate-buffered saline), pH=7.4	50 ml 0.2 M PB, 9 mg NaCl *ad* 1000 ml *aq. bidest*
NGS (Normal goat serum), 10%	500 µl *ad* 5 ml PBS
Blocking solution	NGS 10%, 0.03 % Triton-X100 (Merck)
PFA (Paraformaldehyde), 4%, pH=7.4	40 g PFA powder (Merck) *ad* 1000 ml 0.1 M PB pH-titration with NaOH
Sucrose for cryoprotection, 30%	30 g sucrose *ad* 100 ml PBS
Citifluor for tissue mounting	Citifluor
Fluoromount G for tissue mounting	SouthernBiotech
BDA (biotinylated dextrane amine), MW 10.000, 10%	25 mg BDA (Molecular Probes) *ad* 250 µl physiological saline
DAPI (4,6´-Diamino-2-phenylindole)	1:10.000 (Roche Diagnostics) in PBS

3.2.2 Antibodies

Table 3.3: Primary antibodies.

Antibody	Antigen	Dilution	Supplier
GFAP ms IgG	glial fibrillary acidic protein	1:1000	Millipore
GFAP rbt IgG	glial fibrillary acidic protein	1:1300	Dako
NF rbt IgG	Neurofilament (recognizing NEF-L, NEF-M and NEF-H)	1:1300	Biotrend
hNuc ms IgG	human nuclei	1:500	Millipore
OSP rbt IgG	oligodendrocyte specific protein	1:750	Abcam
S100 rbt IgG	S100 calcium binding protein	1:300	Sigma Aldrich
Tuj1	β-III-Tubulin	1:500	Millipore

Table 3.4: Secondary antibodies.

Antibody	Dilution	Supplier
Anti-ms IgG, Alexa Fluor® 488	1:1000	Invitrogen
Anti-rbt IgG, Alexa Fluor® 564	1:1000	Invitrogen
Anti-rbt IgG, Alexa Fluor®350	1:1000	Invitrogen
Anti-rbt IgG, biotinylated	1:500	Vector Laboratories
Streptavidin, Alexa Fluor® 350	1:500	Invitrogen
Oregon Green® 488	1:500	Invitrogen

3.3 Surgical procedures

3.3.1 Dorsal hemisection

All surgeries were performed using isoflurane anesthesia (Forene (Abbott); 2–3 % in O_2 and NO_2 at a ratio of 1:2). After shaving and disinfection of the skin overlying Th6-Th12, an incision was performed with a scalpel blade exposing the underlying paravertebral muscles. Adipose tissue between the blade bones was removed from the muscles and the transverse processes at Th7-Th10 were exposed. A muscle retractor forced muscle tissue apart followed by a removal of the vertebral arch at Th8. To allow a controlled operation procedure, the spinous processes of Th7 and Th10 were clamped and stabilized using a stereotactic device. The dura mater was opened at the vertebral levels Th8/Th9 using iridectomy scissors via a lateral cut. The dorsal corticospinal tract (CST), the rubrospinal tract (RST) and the dorsal columns were cut with a Scouten wire knife (Bilaney) to the depth of the central canal. Therefore, the guidance canula was stereotactically inserted 1.2-1.5 mm deep into the spinal cord. The dorsal axonal tracts were transected while the opened knife was lifted up. A schematic illustration of the transection procedure is presented in Fig. 3.1. The dorsal hemisection leads to a highly reproducible lesion allowing comparison between different animal groups (Hermanns et al., 2001; Klapka et al., 2005; Schiwy et al., 2009).

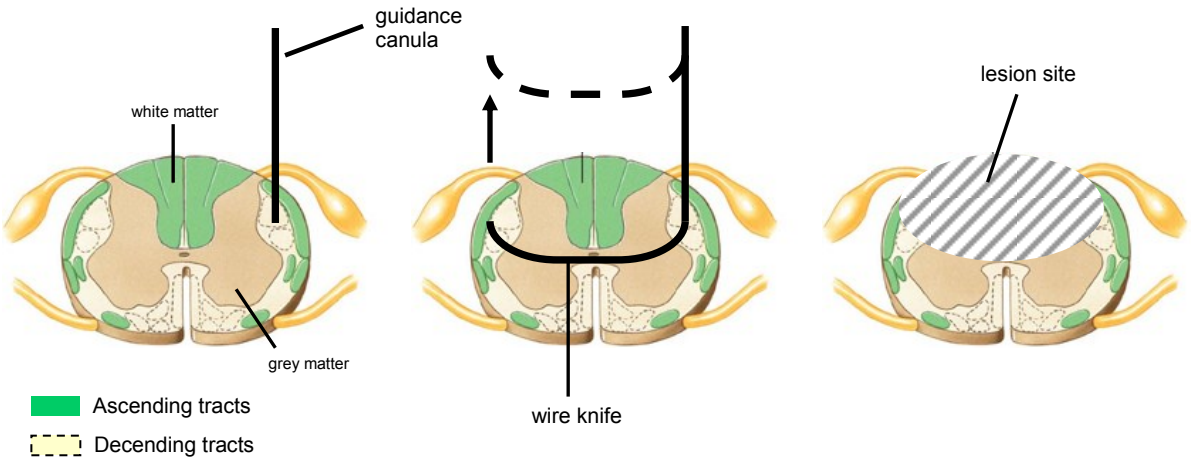

Fig. 3.1: Schematic illustration of the transection procedure with the Scouten wire knife. The guidance canula is stereotactically inserted into the rat spinal cord. The opened wire knife is lifted up cutting the dorsal axonal tracts including the CST and RST (according to http://www.colorado.edu/intphys/Class/IPHY3430-200/image/09-7.jpg, modified)

3.3.2 USSC grafting into the spinal cord

After dorsal hemisection, the dura was sutured and the following USSC grafting was directly performed with a glass capillary 2 mm rostral and 2 mm caudal to the lesion site at 1.2 mm and 0.8 mm depth. 2 µl containing a suspension of 100.000 cells in DMEM were injected slowly within 4 min at each injection site. The animal groups receiving either USSC or DMEM medium (control) were immunosuppressed with cyclosporine A (15 mg/kg s.c., Novartis), beginning one day prior to surgery and thereafter daily for three weeks post-operation. Sham-operated rats received a laminectomy and opening of the dura but no spinal cord injury.

3.3.3 Rostral tracing

For labeling of regenerating fibers extending into the lesion site, anterograde axon tracing was performed rostral to the lesion at Th7 (approximately 3 mm rostral to the lesion site) by microinjections of biotinylated dextrane amine (BDA, 10%, MW

10.000, Molecular Probes) into the spinal cord as described previously (Bamber et al., 2001; Hsu and Xu, 2005). Three injections (0.3 µl each) with glass capillaries attached to Hamilton syringes at 1.5, 1.2 and 0.8 mm depth at the midline and three injections at 1.2, 0.8 and 0.4 mm depth 1 mm bilateral to the CST were carried out at 4 weeks after hemisection. Animals were perfused one week after rostral tracing (in total: 5 weeks post-hemisection).

3.3.4 Post-operative care

Post-operative care included daily oral administration of antibiotics (Baytril®, Bayer Health Care) and manual bladder emptying for one week. 5 ml physiological saline was administered s.c. when the urine was bloody or loss of body weight exceeded 10 g. Further, rats received an analgesic (s.c., Rimadyl, Pfizer) for 2 days. Veterinary assistance was consulted when animals had signs of infection or automutilation.

3.3.5 Animal sacrifice

Deeply anaesthetized rats were transcardially perfused first with ice-cold phosphate-buffered saline (PBS) for 2 min and then 4% paraformaldehyde (PFA, Merck) for 15 min using a perfusion pump (505S, Watson-Marlowe). Spinal cord segments of approximately 5 cm length including the lesion site were collected and post-fixed in 4% PFA for 24 h at 4°C.

3.4 Tissue preparation

After fixation, the lesion containing part of the spinal cord was dissected and washed with PBS. The tissue was cryopreserved in 30% sucrose at 4°C and frozen in Tissue-Tek® (Sakura) on a tissue holder. Tissue was cut into serial 18 µm parasagittal sections using a Cryotome (Leica). Every 10^{th} section was used to characterize USSC with respect to localization, migration and neural differentiation as well as to quantify BDA-traced axons in the lesion area.

3.5 Histological staining protocol

For immunohistochemical stainings, sections were blocked with 10% normal goat serum (NGS, Sigma) and permeabilized with 0.03% Triton X-100 (Merck) for 1h. Incubation with primary antibodies directed to neurofilament protein (recognizing NEF-L, NEF-M and NEF-H, abbreviated as NF) (rabbit, polyclonal, BioTrend, 1:1300 dilution), glial fibrillary acidic protein (GFAP; mouse, monoclonal, Millipore, 1:1000 dilution), GFAP (rabbit, polyclonal, Sigma Aldrich, 1:1500 dilution), S100 (rabbit, polyclonal, Sigma Aldrich, 1:300 dilution), oligodendrocyte specific protein (OSP; rabbit, polyclonal Abcam, 1:750 dilution) and human nuclei (hNuc; mouse, monoclonal, Millipore, 1:500 dilution) was carried out overnight at 4°C. Incubation with hNuc-specific antibody included 10% NGS supplemented with 0.1% Triton X-100. After washing with PBS, secondary antibodies (goat anti-mouse conjugated with Alexa 488, goat anti-rabbit conjugated with Alexa 594 or goat anti-rabbit conjugated with Alexa 350, Invitrogen, 1:1000 dilution, respectively) was applied and incubated for 2h at room temperature. Cell nuclei were labeled with 4,6'-diamidino-2-phenylindoline (DAPI, Roche Diagnostics). Streptavidin Oregon green 488 (Invitrogen, 1:500 dilution) was applied for BDA visualization after rostral tracing. Sections were mounted either with Citifluor (Citifluor) or Fluoromount G (SouthernBiotech). For negative controls in order to exclude nonspecific binding of the secondary antibody, the primary antibody was omitted. Specificity of the primary antibodies was confirmed prior to this study using tissue sections of intact brain and spinal cord. Images were taken either with a fluorescence microscope (Axioplan2, Zeiss) or a LSM 510 confocal microscope (LSM 510, Zeiss). Fig. 4.3A-D and Fig. 4.7B/C are projections from z-stacks assembled by the software LSM image browser (Zeiss).

3.6 Analysis of tissue sections

3.6.1 Quantification of anterogradely labeled fibers

For quantification of BDA-traced fibers, every 10th parasagittal section was stained against BDA (6-8 sections per animal). Only labeled fibers localized within the (GFAP-negative) lesion area were manually counted. Sections were analyzed using a fluorescence microscope (Axioplan2, Zeiss). The number of axon profiles was normalized to the lesion area (axons/mm²) by using Image J software.

3.6.2 Assessment of lesion area and spared tissue

Parasagittal spinal cord sections were stained against GFAP to identify and outline the (GFAP-negative) lesion area. The lesion and spared tissue areas were determined as described previously (Iannotti et al., 2010) with slight modifications. Briefly, from each spinal cord the section with the largest lesion size plus two additional sections taken at a distance of 0.2 mm on each side were used for quantitative evaluation. The area of spinal cord reaching from 1 mm rostral to 1 mm caudal of the midline of the lesion was outlined in composed images of the spinal cord sections to determine the percentile area of the lesion within the outlined region using ImageJ software. Spared tissue area (mm^2) is defined by the total region outlined minus the area of the lesion.

3.7 Behavioral analyses

For behavioral testing, pre-training of 35 rats started four weeks prior surgery in all behavioral tasks. The animals were housed in groups of three with food *ad libitum* throughout the post-surgery testing period of 16 weeks. Five animals died after surgery and/or during the testing period. Both hindlimbs were evaluated separately, because of a slight asymmetry in our Scouten wire knife lesion, which resulted in an enhanced impairment of the left RST in comparison to the right RST (Schiwy et al., 2009).

3.7.1 Open field (BBB) locomotor score

Hindlimb function was evaluated weekly in an open field test using the Basso–Beattie–Bresnahan (BBB) score (Basso et al., 1995). The rats were observed for 4 min by two individuals unaware of the treatment conditions. Rats were placed in a rotund Plexiglas® open field (Febikon) with a diameter of 1 m and a floor which was covered with a black rubber mat. Forelimb-hindlimb coordination was determined separately by analysis of the walking pattern using the CatWalk device and software (Hamers et al., 2001). For this evaluation, every rat had to cross the walkway without any interruption or hitch. A minimum of three correct crossings per animal were required (Koopmans et al., 2005). The CatWalk-based coordination was defined as follows: (i) animals with a regularity index (RI) of 100 % in all three CatWalk crossings were assigned consistent coordination, (ii) animals with a RI of 100% in two of three crossings were scored with frequent coordination and (iii) animals with a RI of 100% in only one crossing were scored with occasional coordination. Animals with a RI lower than 100% in all three crossings were scored as uncoordinated (Koopmans et al., 2005). The 7-point locomotor subscore, which allows evaluation of finer aspects of locomotor control, was also assessed.

3.7.2 Horizontal ladder walking test (gridwalk)

The horizontal ladder walking test (gridwalk) was performed as described elsewhere (Metz et al., 2000; Metz and Whishaw, 2002). Bar distances of the horizontal ladder were irregular and frequently changed to avoid animals to memorize the location of the bars. The walking of each rat was recorded with a conventional video camera and subsequently analyzed in slow motion. Steps and missteps were counted blind for each condition, in five uninterrupted crossings per testing day for each hindlimb separately. A misstep was counted when the foot slipped or fell off the bar. The number of missteps per trial was counted, given as percentile of the total number of steps and averaged for five trials. When a lesioned rat was unable to cross the ladder, a maximum error of 100% was scored. Baseline data were collected one week prior surgery. Animals were tested every week beginning two weeks after

surgery. The horizontal ladder walking error rates were not expressed as a percentage of the baseline scores for each rat, because current and earlier studies demonstrated that uninjured rats make hardly any placement errors on this task (Bolton et al., 2006; Klapka et al., 2005).

3.7.3 CatWalk gait analysis

CatWalk analysis was performed as described elsewhere (Hamers et al., 2001). The animals crossed a horizontal glass runway equipped with a standard CCD camera connected to a PC with the CatWalk software. Animals were tested once a week for at least three uninterrupted runs per animal. Baseline data were collected three days prior to surgery. Coordination and different individual paw parameters were assessed to quantify functional recovery after USSC transplantation. The regularity index (expressed in %) was assessed as a measure of interlimb coordination using the analysis of the step sequences (Vrinten and Hamers, 2003). The following parameters related to single paws were analyzed:

- Relative paw placement (expressed in mm): Distance between the placement of the fore- and hindpaws of the same side of the animal. When the hindpaw is placed upward compared to the forepaw, the value is negative. A positive value implicates that the hindpaw is placed backward compared to the forepaw.
- Base of support of the hindlimbs (BOS HL): Distance between both hindpaws.
- Maximal area (expressed in mm²): Maximal contact area of the paw at the moment of its maximal floor contact during stance.
- Print area (expressed in mm²): Total floor contact reached during the stance phase reflecting the total print size of the paw. A decrease in this parameter can indicate mechanical allodynia (Vrinten and Hamers, 2003).
- Swing speed (expressed in m/s): Speed of the individual paw for two consecutive paw placements.
- Swing duration (expressed in s): Duration of the swing phase of the individual paw.

Values for each parameter (except for relative paw placement) were related to the baseline data collected prior to surgery (relative values).

3.8 Cell cultures

3.8.1 Cell culture media

Table 3.5: Cell culture media.

cell culture media	Composition/Supplier
USSC expansion medium	DMEM (Lonza) 30% FBS (Lonza) 2 mM Glutamine (Gibco, Invitrogen Company) 100 U/ml penicillin/streptomycin (Gibco)
USSC freezing medium	DMEM (Lonza), 20% DMSO (Sigma) 40% FBS (Lonza) 2 mM Glutamine (Gibco) 100 U/ml penicillin/streptomycin (Gibco)
Astrocyte culture medium	DMEM (Lonza) 10% FBS (Lonza) 2 mM Glutamine (Gibco)
Cortical neuron culture medium (N2)	4:1 mixture of DMEM (Lonza) with Ham`s F12 (Invitrogen) 5 ml N2-Mix (Invitrogen) 2 mM Glutamine (Gibco)
DRG culture medium	DMEM Glutamax® (Gibco) 15% FBS (Lonza) 2 mM Glutamine (Gibco)
Coating of coverslips	Poly-D-lysin (PDL), 0.5 mg/ml (Sigma) Laminin, 13 µg/ml (Sigma)
Migration buffer	RPMI medium (Sigma)
Tryspin/EDTA, 1x (0.05%)	Gibco
fixation solution, 4%	1ml Formaledehyde (Merck) *ad* 10 ml PBS

3.8.2 USSC isolation and expansion

Two different USSC populations were used which were provided by the group of Professor Gesine Kögler from the Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Medical Center Düsseldorf. USSC were isolated as described previously (Kögler et al., 2004). The mononuclear cell fraction was obtained by a standard Ficoll-gradient separation from umbilical cord blood followed by ammonium chloride lysis of red blood cells. After two washing steps, the cells were seeded in culture flasks and grown in the presence of dexamethasone. USSC growing as adherent cell colonies were selected. For expansion, USSC were incubated in DMEM (Lonza) supplemented with 30% heat-inactivated fetal bovine serum (FBS, Lonza), 2 mM glutamine (Gibco) and penicillin/streptomycin (100 U/ml, Gibco). USSC were incubated at 37°C in 5% CO_2 in a humidified atmosphere. USSC at passage 5 to 8 were used for all experiments.

3.8.3 Freezing and storage of USSC

Confluent grown USSC were detached from culture flasks using tryspin/EDTA (1x, 0.05%; Gibco) for 3 min at 37°C. Trypsin reaction was stopped in DMEM/10% FBS and cells were centrifuged for 5 min at 1200 rpm. After resuspension in USSC culture medium, cell suspension was gently mixed 1:1 with freezing medium containing DMEM/40% FBS and 20% DMSO (Sigma). Cells were immediately frozen at -20°C for 30 min and then at -80°C over night. Finally, tubes were transferred into liquid nitrogen and stored until thawing.

3.8.4 Preparation of primary astrocyte cultures

Primary astrocytes were isolated from P0-P1 Wistar rats as previously described (Schmalenbach and Müller, 1993). After removal of the skin and skullcap, the brain was taken out and the dura mater was removed. Cerebral cortical tissue was dissected for each brain hemisphere sparing the hippocampus. Meninges were removed from the brain and remaining tissue was fragmented. After resuspension in

DMEM (Gibco) supplemented with 10% FBS and 2 mM glutamine, tissue fragments were subsequently centrifuged at 2000 rpm for 30s. After centrifugation, cells were re-suspended in 10 ml culture medium using a constricted glass Pasteur pipette and passed through a sterile 60 µm nylon gauze. Finally, the cell suspension was centrifuged at 1500 rpm for 5 min. After re-suspension, astrocytes were cultured in T75 standard cell culture flasks in presence of DMEM/10% FBS at 37°C, 98% humidity and 10% CO_2. To remove non-astroglial contaminations, confluent astrocyte cultures were incubated on a shaker at 200 rpm over night. Primary astrocyte cultures were used for 2 passages.

3.8.5 Preparation of conditioned medium

Confluent layers of USSC and primary astrocytes were gently rinsed with PBS and incubated in DMEM Glutamax® supplemented with 15% FBS or in serum-free N2-medium for 48h. Conditioned media of USSC (USSC-CM) and astrocytes (ACM) were collected, centrifuged at 1500 x g for 5 min and then applied to dorsal root ganglia or to cortical neurons in order to test neurite outgrowth. USSC and astrocyte cultures were used only for one preparation of conditioned medium.

3.8.6 Neurite outgrowth assays

3.8.6.1 Preparation of dorsal root ganglia and neurite quantification

Dorsal root ganglia (DRG) were prepared following the protocol of Murphy et al., 1996. DRG were dissected from E12.5 - E14.5 rat embryos. After decapitation, the body was fixed on a dish filled with silicone in ventral position. The muscle tissue surrounding the backbone was carefully removed and the DRG attached to nerve fiber bundles were dissected from fibers and transferred into 0.6% glucose solution. Tissue samples were cut into 2-4 smaller explants and plated on coverslips pre-coated with poly-D-lysin (PDL, 0.5 mg/ml, Sigma) and laminin (13 µg/ml, Sigma). For pre-coating, coverslips were incubated over night at 4°C with PDL, washed three times with PBS and coated with laminin for 2 days at 4°C. After washing with PBS,

culture medium was filled into dishes and equilibrated at 37°C, 98% humidity and 5% CO_2. The DRG explants either received the non-conditioned control medium DMEM Glutamax® (Gibco) supplemented with 15% FBS (Lonza) or the same culture medium after conditioning by exposure to USSC for 48h (USSC-CM_{FBS}). DRG explants were fixed after 24h of incubation and then immunocytochemically analyzed.

For quantification of axon outgrowth, immunofluorescence microscope images were taken from NF-stained DRG. The inner DRG cell cluster and the maximal centrifugal axon spread were outlined using tools of Image J software to measure the area of the core of the explant and region of axonal outgrowth, respectively. For each condition, 10 explants were quantified in three independent experiments (n=30).

3.8.6.2 Preparation of primary cortical neurons

Primary neocortical neurons were isolated from E15 Wistar rats. After removal of the skin and skullcap, the brain was taken out and the dura mater was removed. Cerebral cortical tissue was dissected for each brain hemisphere sparing the hippocampus. Meninges were removed from the brain and the tissue was fragmented. After re-suspension of the tissue fragments in DMEM and centrifugation at 2000 rpm for 30s, the tissue was incubated in 0.05% trypsin (10 min, 37°C). The protease activity was stopped by adding 20 ml DMEM/10% FBS and the tissue was centrifuged at 2000 rpm for 1 min. The pellet was resuspended in 1 ml DMEM using a constricted glass Pasteur pipette. Cell suspension was supplemented with 9 ml DMEM and passed through a 30 µm nylon gauze. Finally, the cell suspension was centrifuged at 1500 rpm for 5 min and the pellet was re-suspendend in 1 ml N2-medium (Bottenstein and Sato, 1979). N2-medium was a 4:1 mixture of DMEM with Ham`s F12 (Gibco, Invitrogen Company) supplemented with 5 ml N2-Mix (Invitrogen) and 2 mM glutamine (Gibco). Cells were seeded on 1 mg/ml PDL and 13 µg/ml laminin pre-coated coverslips in 24-well cell culture dishes at a density of approximately 75,000 cells/ cm^2 for 48h at 37°C, 98% humidity and 10% CO_2. Cortical neurons were incubated either in N2-medium (non-conditioned control

medium), ACM (N2 medium conditioned by astrocytes for 48h) or USSC-CM_{N2} (N2 medium conditioned by USSC for 48h).

3.8.7 Immunocytochemical analysis of cell cultures

For immunocytochemical analyses, DRG explants and cortical neurons were fixed with 4% formaldehyde (Merck), blocked with 10% normal goat serum, permeabilized with 0.03% Triton X-100 for 1h and incubated with the primary antibody overnight at 4°C. The primary antibodies used were anti-neurofilament (NF; rabbit, polyclonal, Biotrend, 1:1300 dilution) and anti-β-III-tubulin (Tuj1; mouse, monoclonal, Millipore, 1:500 dilution). After washing with PBS, incubation with the secondary antibody (goat anti- mouse conjugated with Alexa 488 or goat anti-rabbit conjugated with Alexa 594, Invitrogen) followed for 2h at room temperature. Cell nuclei were stained with DAPI after rinsing with PBS. Coverslips were then mounted in Citifluor (Citifluor).

3.8.8 Under-agarose chemotaxis *in vitro* assay

The under-agarose chemotaxis *in vitro* assay was performed as previously described (Trapp et al., 2008) to precisely characterize the migratory potential of USSC. The method is based upon migration of cells under agarose gel. 1% agarose in RPMI medium (Sigma) was poured into 35 mm plastic dishes. Three 2 mm wide parallel wells were cut 5 mm apart using a template. USSC were added to the central well and allowed to adhere for 2h. The target was added to the left well and the migration buffer serving as control was added to the right well.

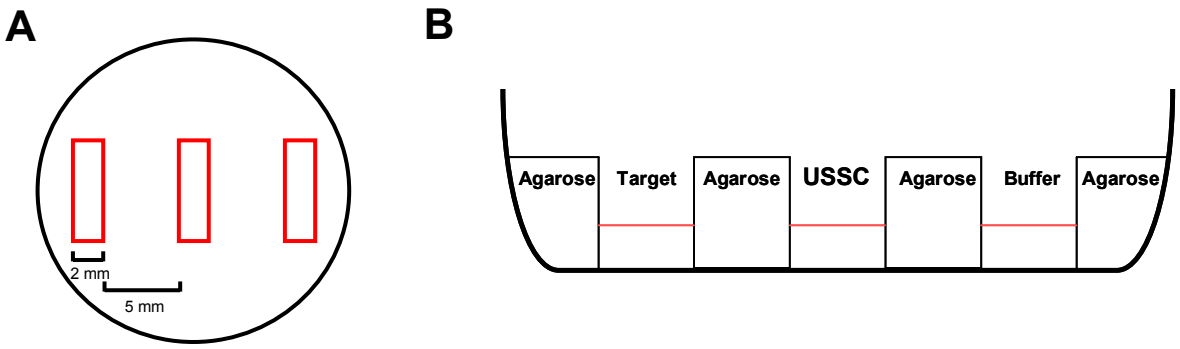

Fig. 3.2: Schematic drawings of the under-agarose chemotaxis *in vitro* assay. (A) Top view. Three parallel wells were cut into the agarose. (B) Side view. USSC were added to the central well, target and buffer were added to the left and right well, respectively.

As targets for USSC migration, extracts (prepared by homogenizing 100 mg tissue/ml of medium in RPMI medium on ice) generated from spinal cord lesions one day post-hemisection and uninjured spinal cord were used. Six hours after incubation, cells were fixed for 30 min in methanol followed by 37% formaldehyde for 30 min. USSC migrating for at least 100 µm into the agarose towards a target were counted. The number of cells migrating towards control buffer (negative control) was subtracted from the number of cells migrating towards the tissue extracts. Data are expressed as fold-increase in cell migration, normalized to the number of cells migrating towards the tissue extract from uninjured spinal cord. For characterization of the chemoattractant responsible for USSC migration, extracts were incubated with 5 µg/ml or 10 µg/ml of anti-HGF antibody (R&D Systems). As control, antibodies unrelated to HGF (R&D Systems) were used at the same concentrations.

3.9 Statistical analysis

All behavioral data were analyzed using the non-parametric Mann-Whitney U test for paired comparison. In order to verify data quality for quantitative analysis of migration, axonal regeneration, DRG neurite outgrowth and spared tissue, significance was assessed by Student's t-test. The experimental groups were considered significantly different at $p < 0.05$. All data are presented as a mean ± SEM.

4. Results

4.1 Migration of USSC in the injured spinal cord

The migratory abilities of endogenous and exogenous stem cells are well known. In addition, the potential to generate various cell types makes stem cells attractive for pre-clinical and clinical investigations to cure and repair injured tissues. Moreover, stem cells might be used as therapeutic delivery vehicles as they can specifically migrate to the site of injury and secrete either trophic factors or specific molecules as they can be genetically modified before transplantation (Aboody et al., 2000; Benedetti et al., 2000; Müller et al., 2006).

In order to investigate the migratory potential of unrestricted somatic stem cells (USSC) from human umbilical cord blood in a CNS disorder, USSC were transplanted into an acute SCI model. Adult rats received a highly reproducible dorsal hemisection injury of the spinal cord at thoracic level Th8 as described previously (Hermanns et al., 2001; Klapka et al., 2005; Schiwy et al., 2009). Immediately thereafter, $2x10^5$ USSC were transplanted at a distance of 2 mm rostral and 2 mm caudal to the site of injury, respectively. To prevent rejection, animals were immuosuppressed with cyclosporine A.

The transplanted human stem cells could be clearly identified by immunostaining using a human specific nuclei antibody (hNuc). One week after transplantation (wpt), USSC were detectable in the lesion center (Fig. 4.1A) which is marked by lack of expression of the axonal marker neurofilament (NF). Of note, large numbers of USSC migrating from the injection site (red arrow, Fig. 4.1A) to the lesion center (white arrow, Fig. 4.1A) could be observed. In contrast, when USSC were transplanted into uninjured spinal cord (SC), the cells did not migrate but remained at the injection site (red arrow, Fig. 4.1B).

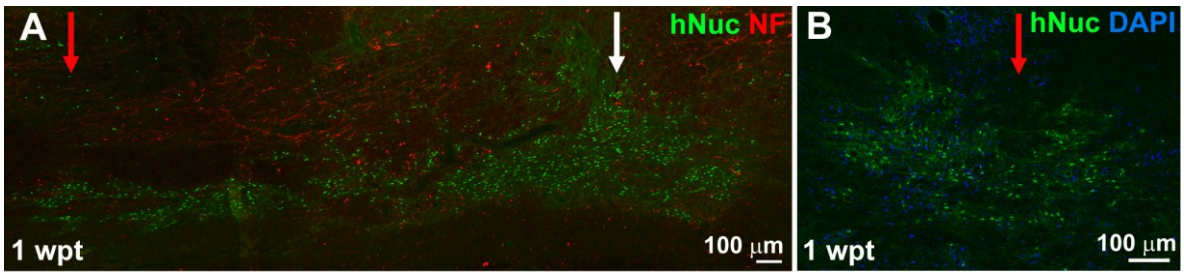

Fig. 4.1: Migration of unrestricted somatic stem cells in lesioned spinal cord. (A) Parasagittal section of the injured SC one week after USSC transplantation. Transplanted USSC were identified immunohistochemically by human specific nuclei antibody staining (hNuc). One week after transplantation, USSC migrate from the injection site (red arrow) to the lesion center (white arrow), which is virtually depleted of neurofilament (NF) immunopositive axons. In contrast, no directed migration occurred after USSC transplantation into the uninjured SC (B).

4.2 Neutralization of HGF-activity leads to inhibition of USSC migration

In an attempt to identify SCI-derived chemoattractants directing USSC towards the lesion site, an under-agarose chemotaxis *in vitro* assay (Nelson et al., 1975; Laevsky and Knecht, 2001) was performed in cooperation with Dr. Thorsten Trapp (Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Medical Center Düsseldorf). In contrast to cell motility assays, this assay allows the formation of a stable gradient of a given chemoattractant. Furthermore, it allows high-resolution imaging and quantification of the motility behavior of individual cells. Tissue extracts from intact and injured SC collected at one day post-surgery were investigated and compared with respect to their chemoattractive activity for USSC. As shown in Fig. 4.2C, USSC were strongly attracted by extracts from the SC lesion, whereas extracts from the uninjured SC only led to a moderate USSC migration (Fig. 4.2B). There was nearly no attraction of the control buffer serving as a negative control (Fig. 4.2A). Quantification revealed that 11-fold more USSC migrated to the lesion extract compared to extracts from uninjured SC after 6h of incubation (Fig. 4.2D).

As shown recently, the hepatocyte growth factor (HGF) is a potential chemoattractant for USSC in a rat model for focal cerebral ischemia (Trapp et al., 2008). To verify the hypothesis that HGF is also involved in USSC migration towards a traumatic spinal cord injury, extracts from the lesion site were incubated with a HGF-neutralizing antibody for 2h. The neutralization of the HGF bioreactivity indeed led to a dose-dependent inhibition of USSC migration to background levels (Fig. 4.2D). Importantly, an unspecific antibody used as control did not show any effect on USSC migration, revealing that the observed inhibition of USSC migration is specific to the neutralizing antibody. Taken together, these results provide evidence that USSC migrate towards SC lesion sites and that HGF is the major chemoattractant for USSC migration.

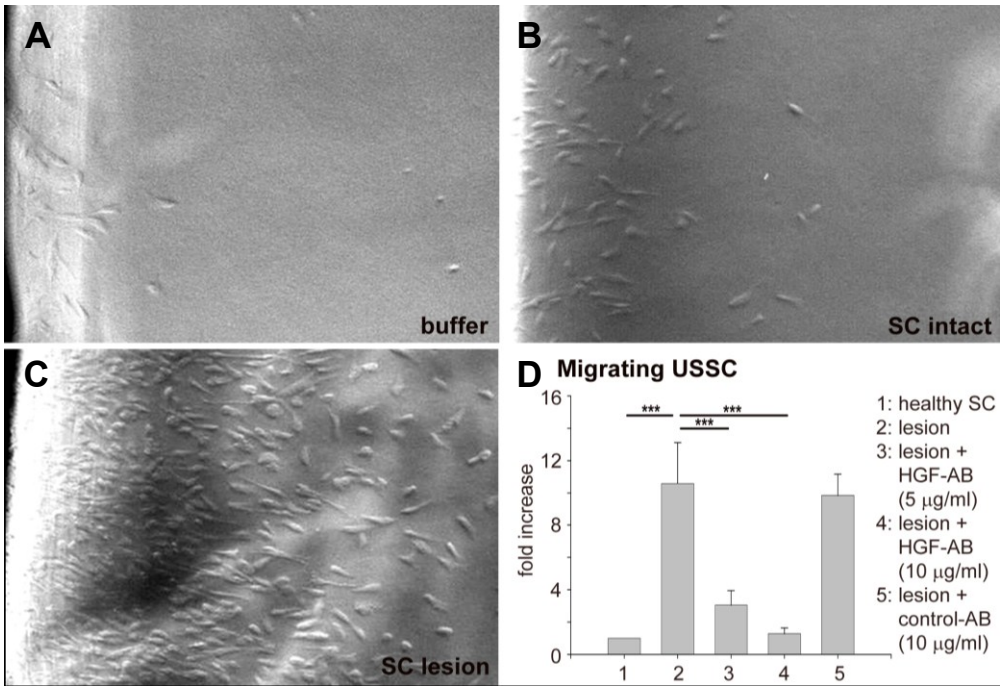

Fig. 4.2: Under-agarose chemotaxis *in vitro* assay. (A-C) Photomicrographs of migrating USSC in the under-agarose chemotaxis *in vitro* assay. USSC were incubated with either control buffer (A), extract from the uninjured SC (B) or extract from the SC lesion site (C) for 6h. The extract from uninjured SC induced only a moderate migration, whereas USSC were strongly attracted by the extract from the SC lesion. Control buffer did not attract USSC. (D) Quantification of migrating USSC. A significantly higher number of USSC migrated to the SC lesion extract compared to extracts from

uninjured SC. Migration could be inhibited to background levels by incubation with an anti-HGF antibody in a dose-dependent manner. Incubation with an unspecific control antibody did not affect the migration. Results derived from four independent experiments are shown as mean values ± SEM. *** $p < 0.001$ (Student´s t-test).

These results were obtained in collaboration with Dr. Thorsten Trapp from the Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Medical Center Düsseldorf, who performed the under-agarose chemotaxis *in vitro* assay.

4.3 USSC lack neural differentiation after transplantation into the injured spinal cord

The potential of stem cells to generate neural cell types has emerged great interest for SCI research. Employing stem cell transplantation has been considered to represent a promising therapeutic approach for SC regeneration. In most studies, replacement of lost cells has been the primary intend for cell transplantation. Pre-clinical investigations have indicated that transplantation of different stem cell types provide beneficial effects on regeneration after SCI due to differentiation into neural phenotypes and cell replacement. As USSC were shown to differentiate into cells with a neural phenotype induced by a composition of growth and differentiation factors *in vitro* (Greschat et al., 2008), the present study investigated the neural differentiation potential *in vivo*. To examine the neural differentiation and subsequent cell replacement potential of USSC *in vivo* after grafting into the injured SC, expression of neuronal and glial marker proteins was immunohistochemically analyzed at 3 wpt. Stainings revealed that USSC neither within nor outside the lesion zone expressed the neuronal marker neurofilament (Fig. 4.3A), glial fibrillary acidic protein (GFAP) (Fig. 4.3B), oligodendrocyte specific protein (OSP) (Fig. 4.3C), or S100 protein (Fig. 4.3D) at three weeks after grafting. Apparently, USSC did not differentiate into neurons, astrocytes, oligodendrocytes or Schwann cells after transplantation into the acutely injured spinal cord.

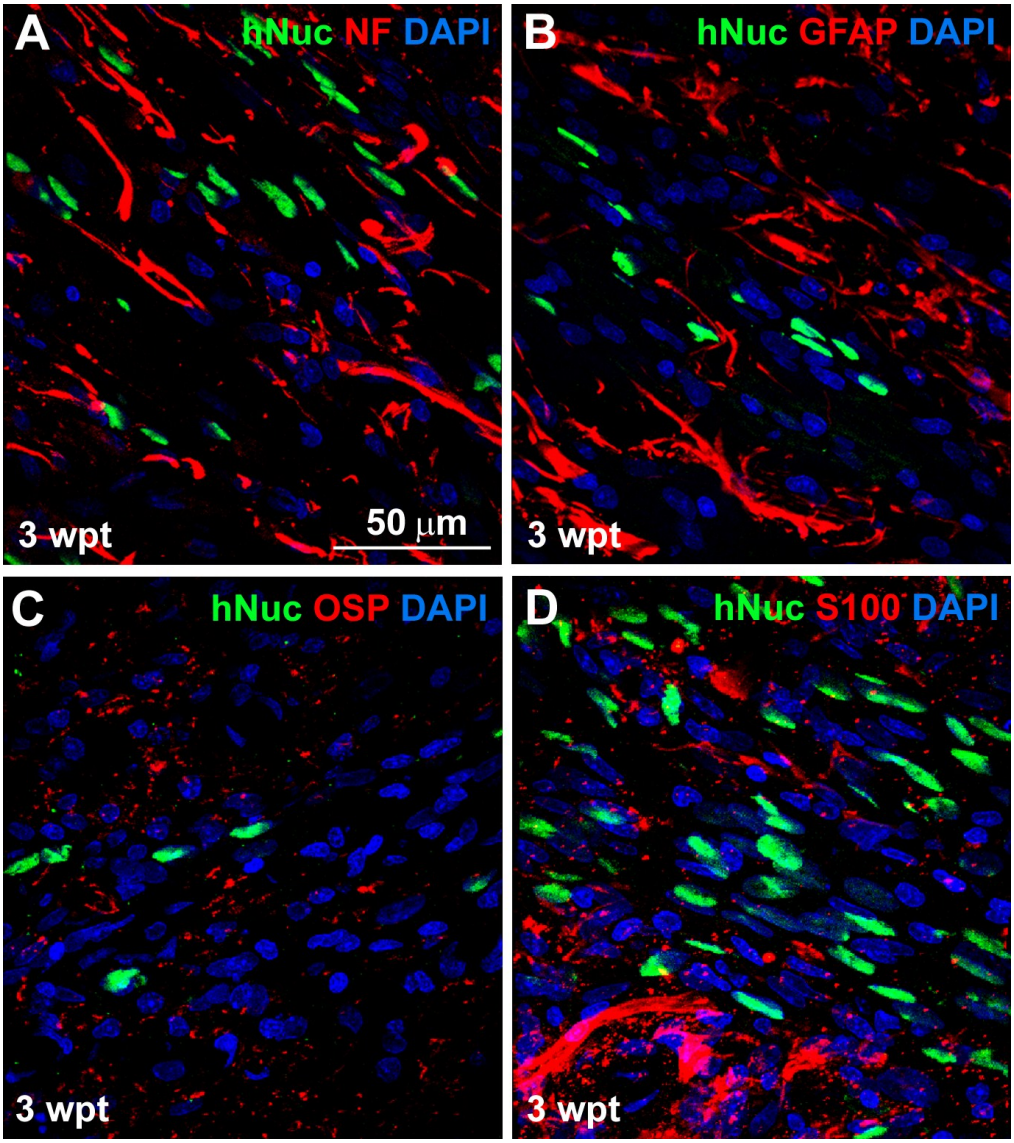

Fig. 4.3: Lack of neural differentiation of USSC grafted into the injured spinal cord. Immunohistochemical stainings of neural marker proteins at three weeks after USSC transplantation into the injured SC. Grafted USSC (hNuc) neither expressed the neuronal protein neurofilament (A), nor the glial proteins GFAP (B), OSP (C) or S100 (D).

4.4 USSC transplantation leads to improved locomotor function after spinal cord injury

Reports on functional recovery after transplantation of MSC derived from bone marrow or hUCB are rather inconsistent. While improvement of sensory and motor activity was reported in some studies (Cizkova et al., 2006; Himes et al., 2006; Cristante et al., 2009), no recovery was observed in others (Neuhuber et al., 2005; Sasaki et al., 2009). Moreover, the majority of the published results reporting functional outcome were based on a single functional test. To assess whether USSC promote long-term locomotor recovery after transplantation into the injured spinal cord, three different locomotor tests were performed in a 16 wpt long-term study. Rats were tested under blinded experimental conditions, starting at 1 wpt with the open field BBB locomotor score for assessment of the overall locomotor behavior. The forelimb-hindlimb coordination was assessed separately using CatWalk gait analysis (Koopmans et al., 2005). Three correct crossings of the walkway per animal were required. Rats with a regularity index (RI) of 100 % in all three CatWalk crossings were assigned consistent coordination, animals with a RI of 100% in two of three crossings were scored with frequent coordination and animals with a RI of 100% in only one crossing were scored with occasional coordination. Animals with a RI lower than 100% in all three crossings were scored as uncoordinated (Koopmans et al., 2005). The 7-point locomotor subscore, which allows more detailed evaluation of aspects of locomotor control was also assessed. Horizontal ladder testing to analyze fine locomotor performance and coordination and CatWalk gait analysis to evaluate individual paw parameters were carried out starting at 2 wpt. Both hindlimbs were analyzed separately in all three locomotor tests.

4.4.1 Open field BBB locomotor scoring

As shown in Fig. 4.4A, the BBB score initially decreased to scores between 8 and 9 at 1 wpt both in the USSC-transplanted and the control group as a consequence of the operation procedure. Within 3 weeks, both groups rose to BBB scores of approximately 11, but the score did not improve further due to the lack of

coordination as revealed by CatWalk gait analysis (regularity index, Fig. 4.4B). The RI decreased after dorsal hemisection, indicating impairment of interlimb coordination. Sham operated animals were not impaired by the surgical procedure and showed a constant BBB score of 21 (data not shown).

Fine locomotor control as assessed by the 7-point BBB subscore revealed that both hindlimbs improved in the USSC-transplanted group, reaching significance in the right hindlimb starting at 6 wpt (Fig. 4.4C,D). Improved fine locomotor control in the right hindlimb of USSC-transplanted rats remained significantly enhanced until, at least, 16 wpt. At this time point, the right hindlimb of USSC-transplanted rats reached a mean subscore that was 2.6 points higher than in control rats. At the end of the study, the score for the left hindlimb of USSC-transplanted animals was approximately 0.8 points higher than in control rats (Fig. 4.4C,D). Sham animals showed a constant locomotor subscore of 7 (data not shown).

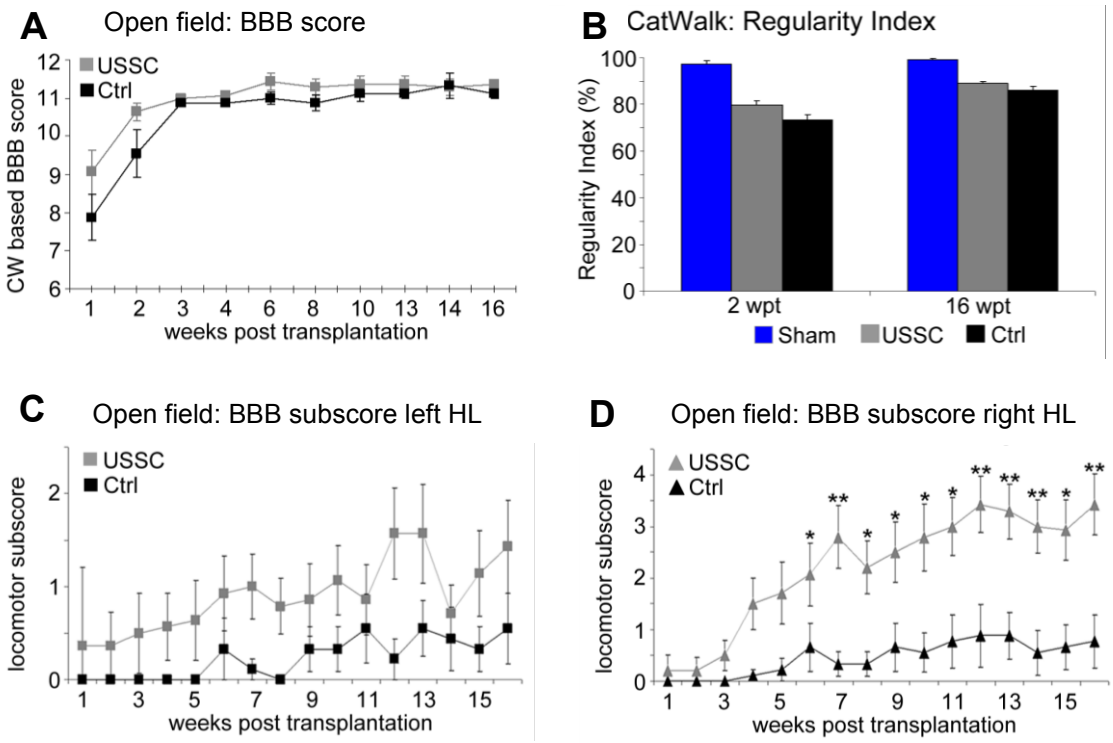

Fig. 4.4 BBB score, subscore and regularity index after USSC transplantation. Time course of locomotor performance in a long-term study comparing an USSC-transplanted animal group (n=14), a non-grafted control group (n=9) and a Sham group (n=7). (A) Overall locomotor behavior assessed by

open field BBB locomotor scoring with a separate analysis of the coordination by CatWalk gait analysis. The CatWalk-based BBB locomotor score revealed no significant differences between the USSC-transplanted and the control (ctrl) group due to the lack of coordination (Sham group not shown). (B) The "Regularity Index" assessed by Catwalk gait analysis decreased after dorsal hemisection, indicating impairment of interlimb coordination. Up to 16 wpt, USSC-transplanted animals showed similar interlimb coordination as the control group. (C,D) The locomotor subscore for the right hindlimb (HL) revealed that USSC-transplanted rats have a significantly improved fine locomotion compared to the control (ctrl) group (D), whereas for the left hindlimb (C) no significant difference between the USSC-transplanted and the control group was observed. Results are shown as mean ± SEM. * $p < 0.05$, ** $p< 0.01$ (Mann-Whitney U test).

4.4.2 Horizontal ladder walking test

The horizontal ladder walking test was performed to assess deficits in descending fine motor control and impairment of forelimb-hindlimb coordination after SCI. Rats had to cross the horizontal ladder with irregular bar distances for at least 5 times per testing day. The relative error rate per hindlimb was expressed as number of missteps (slips and falls) per total number of steps given in percent. While the uninjured sham animals performed close to zero placement errors on this task, dorsal hemisection caused major deficits as reflected in the high error rate of both right and left hindlimb (Fig. 4.5A,B). Interestingly, USSC-transplanted animals made significantly and consistently less missteps with both hindlimbs as compared to the control group (Fig. 4.5A,B). At 6 wpt, USSC-transplanted rats showed an error rate ranging from 43% ± 3 for the right hindlimb (rHL) to 64% $\pm$ 4 for the left hindlimb (lHL), respectively, whereas the error rate was much higher in the rHL (66% $\pm$ 4) and lHL (82% $\pm$ 3) of the control animals. The significantly improved locomotor performance of the USSC-transplanted group remained until the end of the study at 16 wpt, reaching reduction in placement errors compared to controls of 23% (rHL) and 12% (lHL), respectively.

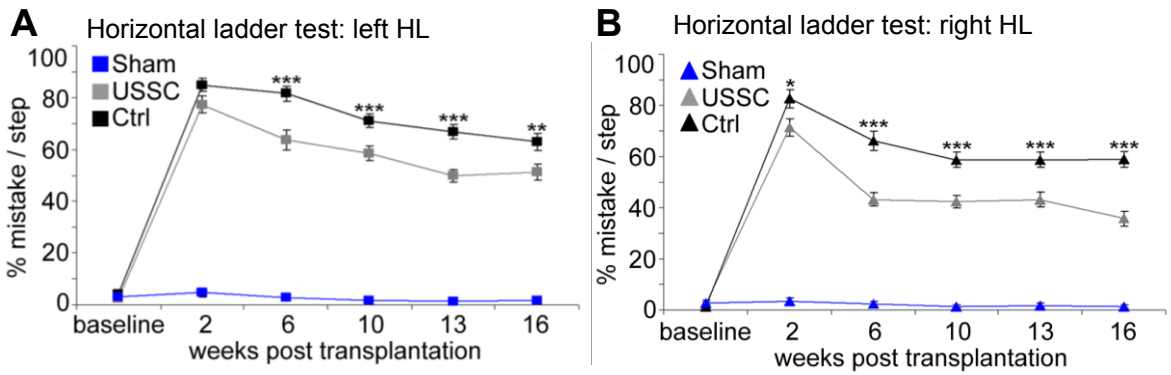

Fig. 4.5 Horizontal ladder walking test. Horizontal ladder walking test revealed significant functional recovery of the USSC-transplanted group for both left (A) and right hindlimb (B) compared to non-grafted controls. Missteps of sham animals were very rare. Results are shown as mean ± SEM. ** p< 0.01, *** p < 0.001 (Mann-Whitney U test).

4.4.3 Catwalk gait analysis

CatWalk gait analysis was performed for quantitative assessment of interlimb coordination (regularity index), different static (relative paw placement, base of support, maximal print and paw area) and dynamic (swing speed and swing duration) gait parameters for individual paws. In Fig. 4.6A-F data of the first (2 wpt) and the last testing day (16 wpt) after surgery are shown. All values, except relative paw placement, were normalized to the baseline, which was measured prior to surgery (relative values).

The Catwalk parameter “relative paw placement” defines the distance between the placement of the ipsilateral fore- and hindpaws. While rats place their hindpaws at or close to the previous position of the forepaw, this ability was largely lost after dorsal hemisection as indicated by the large distance between the paw placement positions, respectively (Fig. 4.6A). Importantly, already at 2 wpt USSC-grafted animals placed their right hindpaw significantly closer to the previous position of the right forepaw than control animals. At 16 wpt, both hindlimbs were placed significantly closer to the previous forepaw positions in grafted animals than in controls. As an additional static gait parameter, the “base of support” (BOS HL) was

analyzed to assess, e.g., trunk stability (Hamers et al., 2006). BOS HL indicates the distance between both hindpaws. As shown in Fig. 4.6B, BOS HL was increased in both animal groups at two weeks after dorsal hemisection. However, in the control group BOS HL further increased, whereas it remained constant in USSC transplanted animals resulting in a significant difference between the two animal groups at 16 wpt (Fig. 4.6B). Moreover, while the contact area of the hindpaws to the floor was reduced in both animal groups, as reflected by the decreased size of the "paw print area at maximal contact", the maximum paw print area of the USSC-transplanted animals was significantly higher than in controls (Fig. 4.6C). In addition, the total paw size as indicated by the parameter "print area" was significantly larger for both hindpaws of the grafted animal group (Fig. 4.6D). The dynamic gait parameters "swing speed" (Fig. 4.6E) and "swing duration" (Fig. 4.6F), which normally show only little variation between individual animals (Hamers et al., 2006), were affected in both control and USSC-transplanted groups. Compared to sham animals, the swing speed was markedly reduced in the hindlimbs of both animal groups, whereas the relative swing speed remained significantly lower in the non-transplanted control group than in the USSC-grafted rats (Fig. 4.6E). On the other hand, the swing duration, which was low in sham animals, increased after SCI, but largely recovered in both grafted and control groups within 16wpt (Fig. 4.6F). Of note, in USSC transplanted animals both dynamic gait parameters, "swing speed" and "swing duration", were marked by a higher degree of recovery than in the control group.

Taken together, the behavioral data demonstrate that USSC transplantation significantly improved the locomotor performance compared to spinal injured animals lacking the stem cell graft.

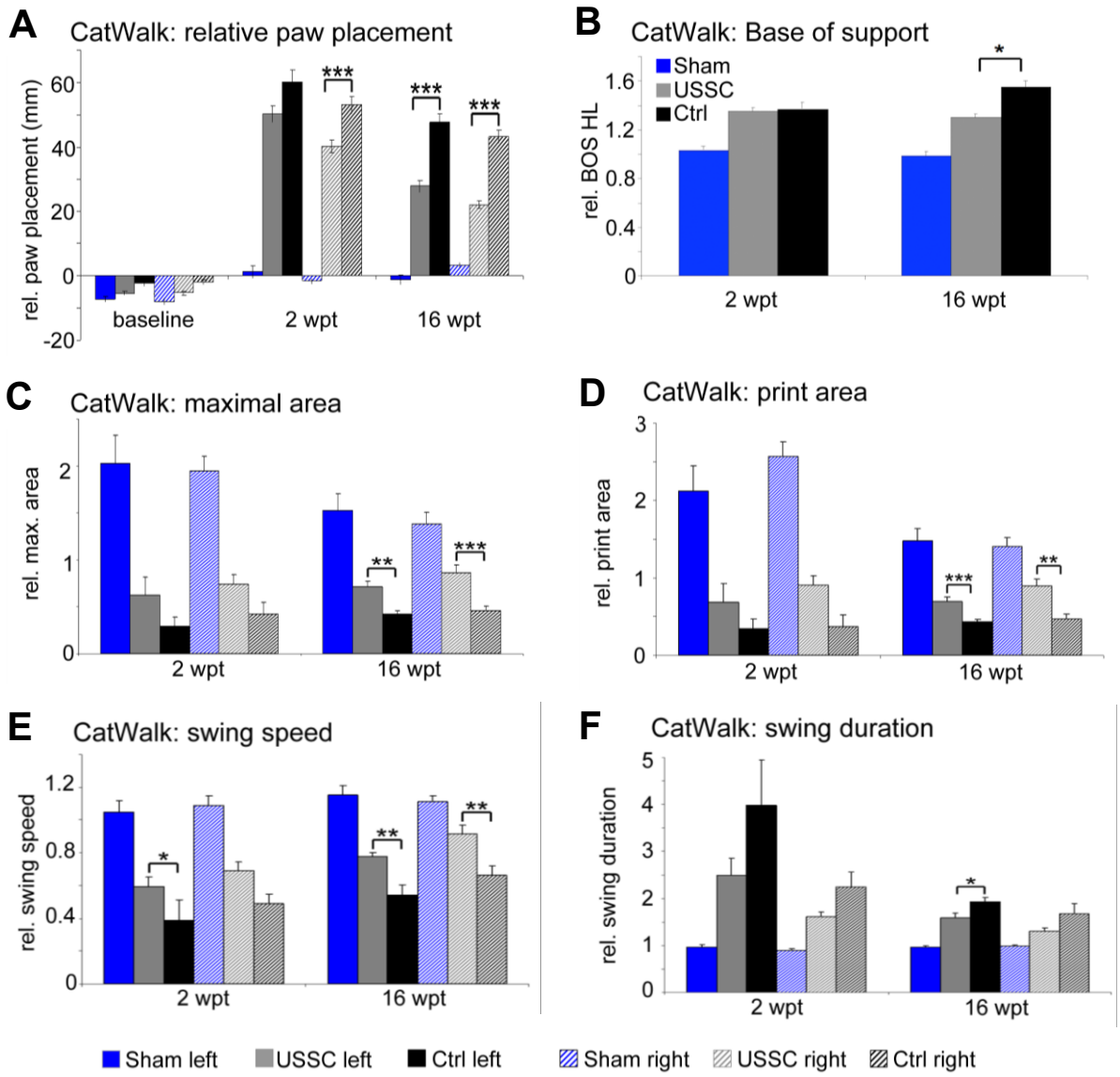

Fig. 4.6: Catwalk gait analysis. The static parameters “relative paw placement” (A), “base of support” (B), “maximal paw area” (C) and “print area”(D) as well as the dynamic parameters “swing speed” (E) and “swing duration” (F) were affected in both groups by SCI. USSC transplantation resulted in significantly improved paw placement and improved hindlimb base of support (BOS HL), enhanced contact of the paw with the floor, a faster swing speed and shorter swing duration at 16 wpt. Results are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Mann-Whitney U test).

4.5 Axonal regeneration after USSC transplantation

4.5.1 Neurofilament-positive fibers in the lesion site

Evidence was provided revealing that CNS axons have the intrinsic capacity to regenerate (David and Aguayo, 1981, Benefey and Aguayo, 1982). Hence approaches are under investigation to improve axonal regeneration. To investigate whether USSC transplantation has beneficial effects on axonal regeneration, immunohistochemical stainings against the axonal marker neurofilament (NF) and anterograde tracing experiments were performed. Immunohistochemical stainings revealed that USSC survived for at least three weeks after transplantation. Surviving cells were mainly confined to the injury site (Fig. 4.7A). Beyond the lesion site, only a few cells could be identified between the injection site and the lesion center. A high number of NF-positive fibers was found in the lesion center after USSC grafting. In close proximity to the grafted cells, numerous elongated neurofilament-positive fibers were present (Fig. 4.7B). In contrast, in control rats lacking USSC grafts, neurofilament labeling was virtually absent in the lesion area (Fig. 4.7C).

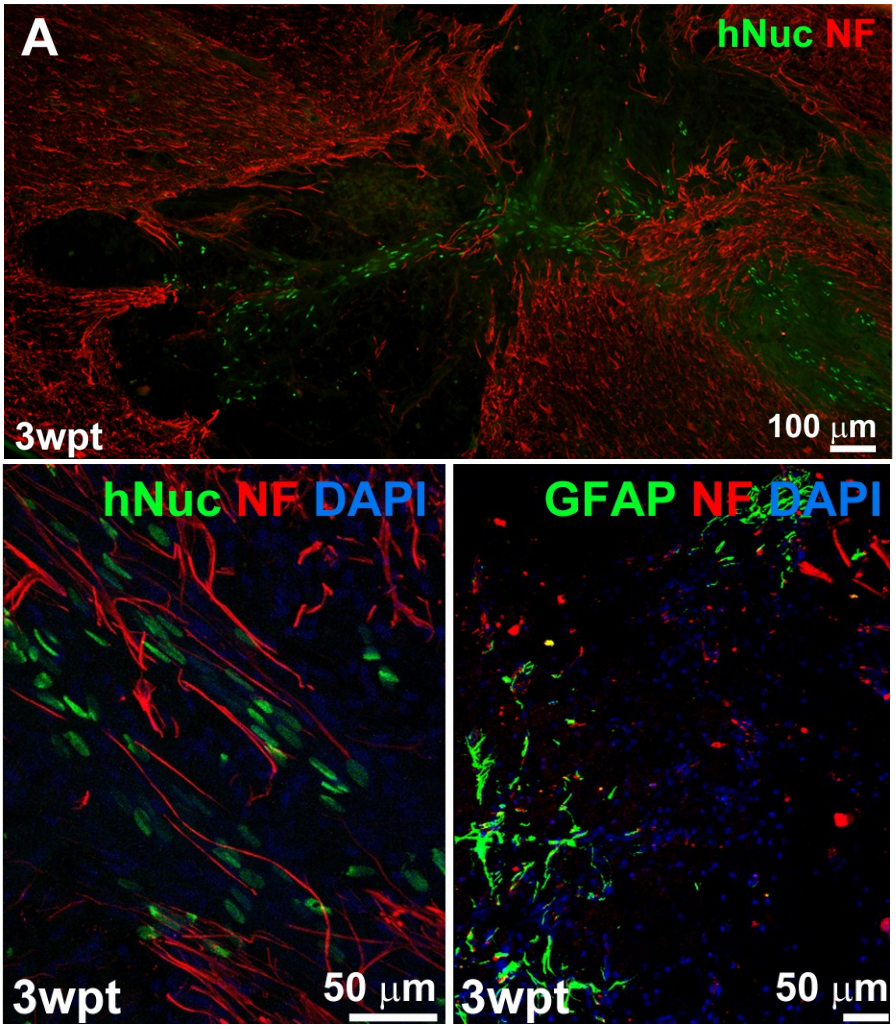

Fig. 4.7: Enhanced axonal regeneration after USSC transplantation. (A) Immunohistochemical staining of a parasagittal section three weeks after USSC transplantation. USSC (hNuc) were identified in the lesion center in close proximity to numerous neurofilament (NF) positive fibers. (B) Close-up view of elongated axons in the USSC-rich lesion core. (C) Neurofilament staining was nearly absent in the GFAP-negative lesion zone of control rats.

4.5.2 Quantitative analysis of BDA-traced axons

The promising finding that USSC enhance the amount of NF positive fibers in the lesion area led to further investigations of the regenerative axon growth promoting activity of USSC. As axon debris could be false-positively stained for neurofilament, rostral tracing experiments were performed four weeks after USSC transplantation.

Descending axons rostral to the lesion were traced at Th7 by anterograde labeling with BDA one week prior sacrifice, allowing the identification of regenerating fibers of different tracts invading the lesion zone. Therefore, staining of traced axons in control animals reflects the spontaneous axon regeneration capacity at the analyzed time point. Immunohistochemical staining 5 wpt revealed that numerous BDA-labeled fibers were grown in the lesion center (the lesion border is indicated by a red line) in USSC-grafted rats (Fig. 4.8A), whereas BDA positive fibers rarely grew into the lesion zone of control rats (Fig. 4.8B).

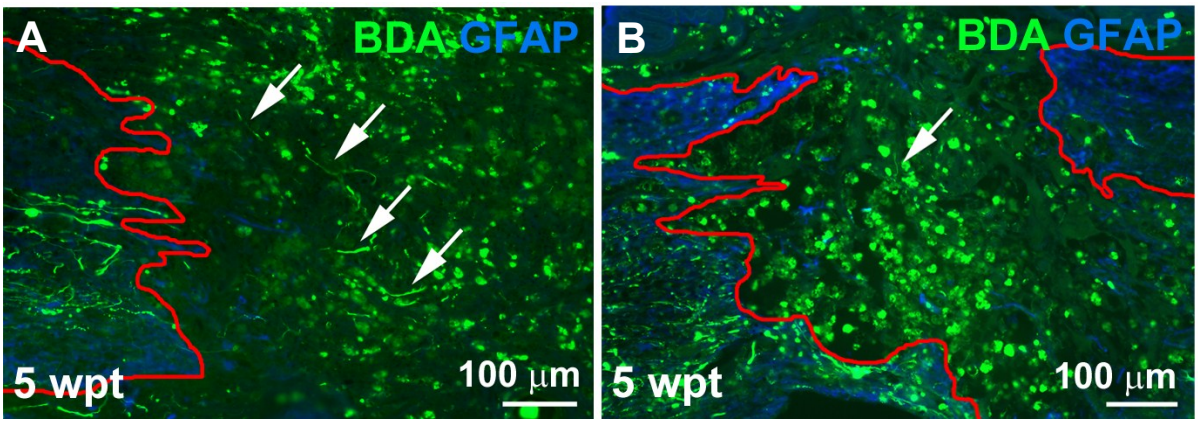

Fig. 4.8: BDA labeling of regenerating axons. (A,B) Immunohistochemical stainings for anterogradely BDA traced axons of parasagittal sections five weeks after USSC transplantation (A) and control grafting (B). The lesion border is indicated by a red line. USSC transplantation increased the extent of axonal regeneration within the lesion site. Arrows indicate BDA traced axons. BDA was visualized with OregonGreen® 488.

The qualitative finding that USSC promote regenerative axon outgrowth was verified by quantitative image analysis determining the density (axon per mm²) of traced fibers in the GFAP-negative lesion center. Only BDA-labeled structures which could clearly be identified as fibers were manually counted as the high background labeling in the lesion area of BDA-traced animals made it impossible to analyze the sections automatically. Cellular debris and macrophages have been positively stained, often with a higher intensity than the fibers had. As shown in Fig. 4.9, transplantation of USSC significantly enhanced the number of regenerating axons

within the lesion area by 2.3-fold (*** p< 0.001) as compared to control animals. These data provide evidence that USSC promote axonal regeneration after transplantation into the injured SC.

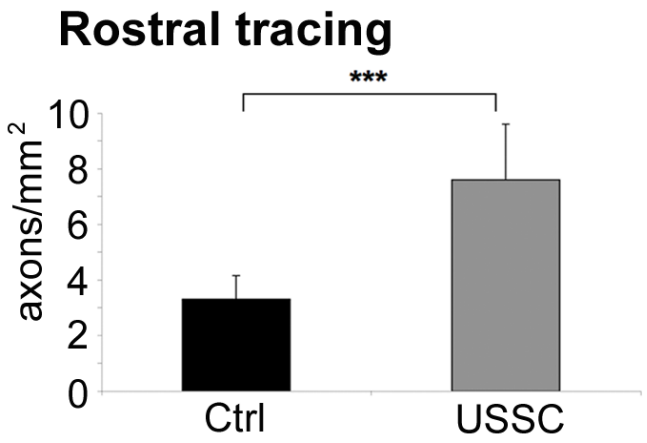

Fig. 4.9: Quantification of the density of traced axon profiles in the lesion zone 5 wpt. BDA-labeled fibers were manually counted and normalized to the lesion area (axons/mm²). USSC grafting resulted in a significantly larger number of regenerating axons as compared to control (ctrl) animals. Results derived from five animals per group are shown as mean ± SEM. *** p < 0.001 (Student´s t-test).

4.6 Enhanced neurite outgrowth of primary neuronal cultures after incubation with USSC-conditioned medium

4.6.1 Dorsal root ganglia explants

To examine whether factors secreted by USSC could account for the neurite growth promoting activity, two different *in vitro* neurite outgrowth assays were performed. Dorsal root ganglia (DRG) explants prepared from embryonic rats were incubated with USSC-conditioned medium (USSC-CM_{FBS}) for 24h. USSC-CM_{FBS} was freshly collected from confluent USSC exposed to DMEM Glutamax®/15% FBS for 48h and subsequently centrifuged to remove cell debris. Axons were visualized immunocytochemically by anti-neurofilament staining. After incubation of DRG with USSC-CM_{FBS}, an extensive radial outgrowth of longer neurites could be observed

(Fig. 4.10A), compared to DRG cultures receiving non-conditioned control medium (Fig. 4.10B). For quantification, the inner DRG cell cluster and the maximal centrifugal axon spread were outlined using tools of Image J software to measure the area of the core of the explant and the region of axonal outgrowth, respectively. Quantitative image analysis confirmed that incubation of DRG with USSC-CM_{FBS} significantly enhanced the neurite outgrowth by two-fold (*** $p < 0.001$) (Fig. 4.10C). Notably, the central cores of the DRG ganglia were almost identical in size in both test and control samples.

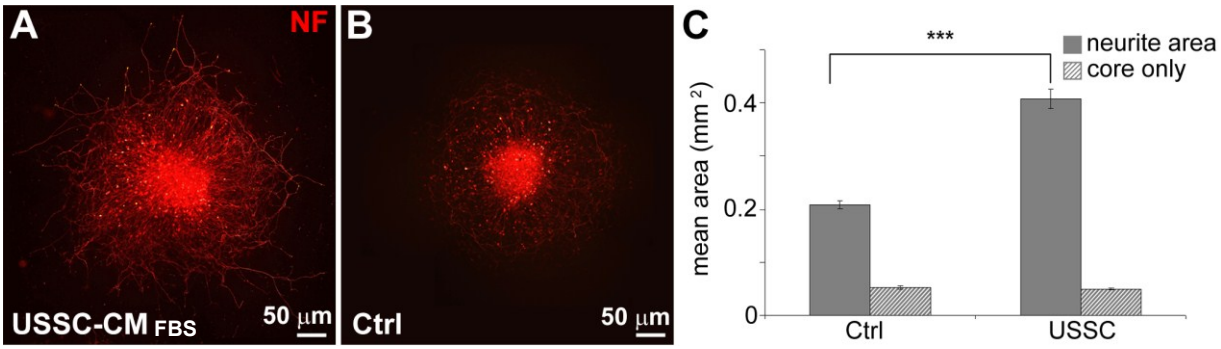

Fig. 4.10: USSC-conditioned medium (USSC-CM_{FBS}) stimulates neurite outgrowth of DRG explants. (A) Immunocytochemical staining of an embryonic dorsal root ganglion (DRG) explant incubated for 24h with USSC-CM_{FBS}. Numerous long neurofilament-positive axons grew out radially, whereas DRG incubated in control medium (ctrl, B) showed less neurite outgrowth. (C) Quantification of the area covered by neurofilament-positive axons revealed that the area of neurites grown in USSC-CM_{FBS} was significantly larger compared to the control. The central core area, however, remained at equal size in both conditions. Results derived from three independent experiments are shown as mean ± SEM. *** $p < 0.001$ (Student´s t-test).

4.6.2 Primary cortical neurons

In a second *in vitro* assay cortical neurons were prepared from embryonic rats and incubated with USSC-CM_{N2} for 48h (Fig. 4.11A). USSC-CM_{N2} was freshly collected from confluent USSC exposed to serum-free N2 medium for 48h and subsequently centrifuged to remove cell debris. Negative controls received serum-free N2 medium only which is free of unknown factors of external sources. As positive control,

astrocyte-conditioned medium (ACM) (Fig. 4.11C) was used, which is known to promote survival and extensive neurite outgrowth (Müller et al., 1984) of cultured CNS neurons. Neurites were visualized immunocytochemically by anti-β-III-tubulin (Tuj1) staining. In the presence of USSC-CM_{N2}, neurite outgrowth and formation of a dense neurite network was stimulated (Fig. 4.11A), whereas nearly all neurons died in non-conditioned control medium (Fig. 4.11B). The effect of USSC-CM_{N2} on neurite outgrowth of cortical neurons was very similar to that of ACM.

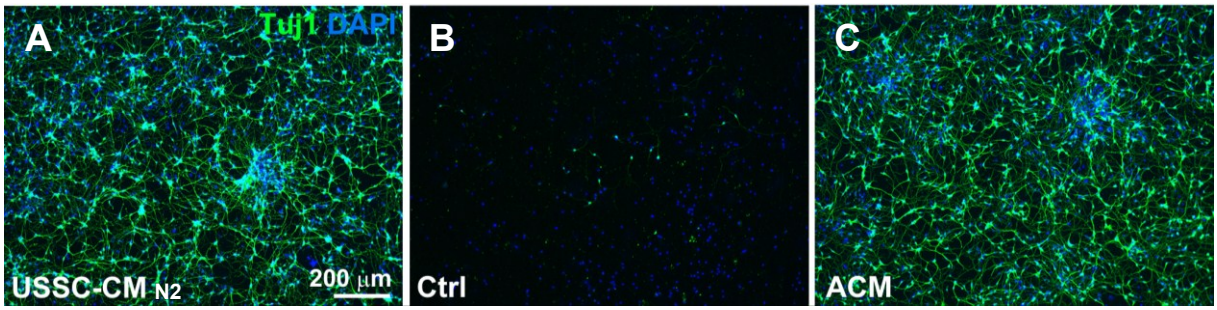

Fig 4.11: USSC-conditioned medium (USSC-CM_{N2}) stimulates neurite outgrowth of cortical neurons. (A-C) Immunocytochemical staining of the neuronal protein β-III-tubulin (Tuj1) on cortical neurons prepared from embryonic rats incubated with USSC-CM_{N2} (A), control medium (B) and ACM (C) for 48h. Note the very similar dense neurite network developing in USSC-CM_{N2} and ACM, whereas only a very small number of neurons survived in non-conditioned control medium. Cell nuclei were stained with DAPI.

4.7 Influence of USSC transplantation on the lesion size

After SCI, cell death persists for weeks resulting in an increase in lesion size and decrease in spared tissue. Different stem cell populations have been shown to reduce tissue loss and enhance tissue sparing after transplantation into the injured spinal cord as they could provide neurotrophic support to neurons and glia (Yoshihara et al., 2007; Kim et al., 2009). In order to investigate whether USSC transplantation reduces tissue loss, parasagittal sections were stained with an anti-GFAP antibody to visualize the (GFAP-negative) lesion core at 16 wpt (Fig. 4.12). This revealed that animals treated with USSC have a smaller lesion size (yellow line,

Fig. 4.12A) than controls (Fig. 4.12B). The lesion size (Fig. 4.12C) and the area of spared tissue (Fig. 4.12D) were examined and quantified using Image J software. Quantification showed that in USSC-transplanted rats the relative lesion size was significantly smaller (24.7% ± 5.2 *vs* 43.4% ± 4.2; ** p< 0.01). At the same time, the area of spared tissue was significantly larger (2.1 mm² ± 0.33 *vs* 1.15 mm² ± 0.1; ** p< 0.01) than in the control group. These data demonstrate that USSC treatment reduces tissue loss after transplantation in a model of acute SCI.

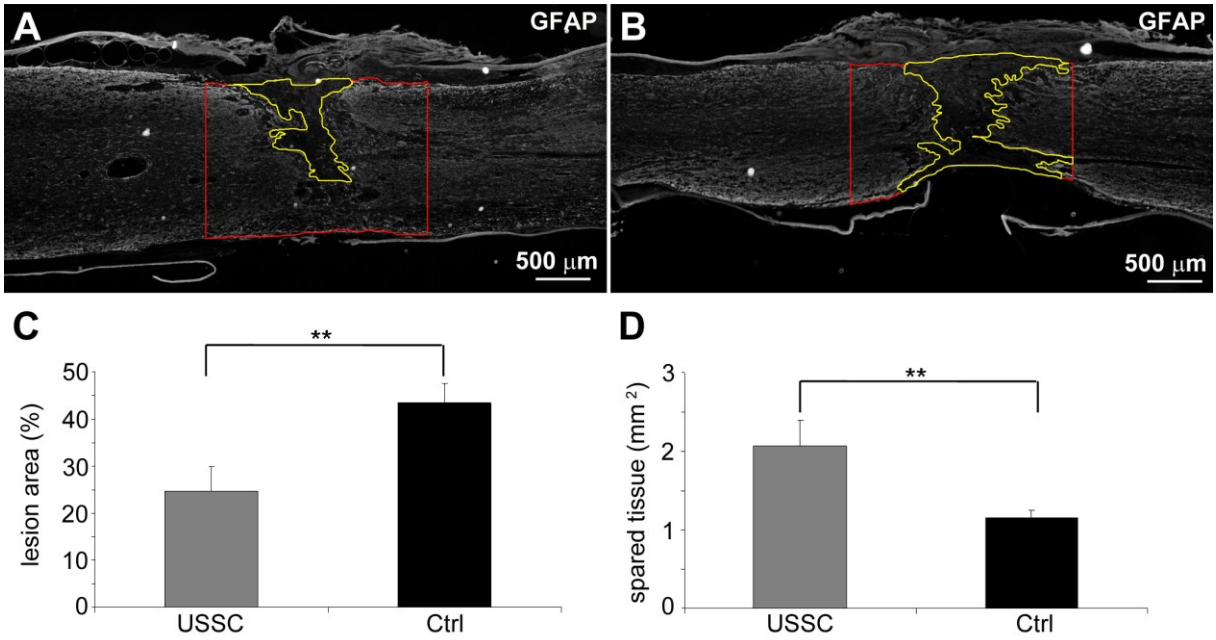

Fig. 4.12: USSC transplantation prevents loss of spinal cord tissue. (A,B) Parasagittal spinal cord sections stained against GFAP to identify the GFAP-negative lesion center at 16 wpt. The yellow line indicates the GFAP negative lesion area; the red line indicates the total spinal cord area. Compared to the USSC-grafted rat (A), the control animal (B) has a larger size of the lesion and a smaller region of spared tissue. Quantification of the lesion area (C, in percent) and the area of spared tissue (D, in mm^2) shows that USSC transplantation significantly improves tissue sparing. Results are shown as mean ± SEM; **** p< 0.01 (Student´s t-test).

5. Discussion

Traumatic spinal cord injury (SCI) results in break down of the blood-spinal barrier, influx of inflammatory cells, activation of glial cells, loss of oligodendrocytes and axonal degeneration leading to permanent motor and sensory deficits. Transplantation of stem cells has been considered as a potential therapeutic strategy to cure SCI. Several pre-clinical studies have indicated that transplantation of different stem cell types provide beneficial effects on regeneration after SCI. In most studies, replacement of lost cells has been the primary intend for cell transplantation. On the other hand, beneficial effects have been demonstrated without any lineage-specific differentiation or obvious cell replacement. The mechanism here appears to be indirect, as transplanted cells might release growth promoting factors, modulate the inflammatory response, protect the endogenous tissue or provide a permissive environment for regenerating axons.

Up to now, stem cells of human sources grafted into the injured spinal cord mostly included barely defined heterogeneous mesenchymal stem cell populations derived from bone marrow or umbilical cord blood (Cizkova et al., 2006; Himes et al., 2006; Cristante et al., 2009; Pal et al., 2009). Still, reports on functional recovery are rather inconsistent. While improvement of sensory and motor activity was reported in some studies (Cizkova et al., 2006; Himes et al., 2006; Cristante et al., 2009), no recovery was observed in others (Neuhuber et al., 2005; Sasaki et al., 2009).

In the present thesis, the well-defined stem cell population of USSC from human umbilical cord blood was transplanted into a rodent model of acute SCI. Several aspects have been investigated in this thesis. First, the survival time, migratory behavior and neural differentiation potential of USSC after transplantation were analyzed. Second, their influence on axonal regeneration, lesion size and protection from spinal tissue loss was studied. Finally, the functional outcome using three different locomotor tasks was investigated. Grafting of USSC into the vicinity of a dorsal hemisection injury at thoracic level Th8 resulted in hepatocyte growth factor-directed migration and accumulation within the lesion area, regenerative axon growth, tissue sparing and significant functional locomotor improvement. To

accomplish the beneficial effects, neither neural differentiation nor long-lasting persistence of the grafted human stem cell appears to be required. The secretion of neurite outgrowth promoting factors *in vitro* further suggests a paracrine mechanism underlying the beneficial effects of USSC in SCI.

This thesis demonstrates, for the first time, that transplantation of a well-defined somatic stem cell population derived from hUCB into acute spinal cord injured rats is an effective strategy to promote regeneration after SCI.

5.1 Migration of USSC in an acute lesioned spinal cord

Homing of circulating, resident or transplanted stem cells to the site of injury is well established. Following CNS injury, various types of stem cells were shown to migrate to pathologically altered tissue where they contribute to tissue regeneration (Aboody et al., 2000; Imitola et al., 2004; Hill et al., 2004; Robin et al., 2006; Rosenkranz et al., 2010). In the case of regeneration of the CNS, migration of transplanted stem cells is important to facilitate either remyelination of axons, tissue preservation or axonal regeneration. The understanding of molecular mechanisms of migration is essential in the development of new therapeutic strategies for tissue regeneration after injury (Enomoto et al., 2003).

In the present study, transplantation of USSC into close proximity to an acute traumatic SCI led to massive migration to the lesion area where the stem cells accumulate (see Fig. 4.1). This observation is consistent with the notion that the migration of stem cells is enhanced after transplantation into lesioned spinal cord (Franklin et al., 1996; Parr et al., 2008). In contrast, when USSC were transplanted into a healthy SC, USSC remained in the injection site. This finding is in accordance with recent studies, where various stem cell populations were shown to be localized in the injection site when transplanted into healthy tissue (Lee et al., 2003; Enomoto et al., 2003). The results presented here thus indicate that USSC are strongly attracted by the SC lesion site. For detailed analysis of the migratory behavior in response to SCI, an under-agarose chemotaxis *in vitro* assay was performed. Measurement of chemotaxis under agarose offers several advantages over the

Boyden chamber method including the simplicity of use for quantitative analyses and the formation of a stable gradient of the chemoattractant. Furthermore, the technique is free from irregularities encountered using the Boyden technique attributable to uneven fluid levels in the two chambers or to variations in filter batches (Nelson et al., 1975). In the present study, under-agarose chemotaxis *in vitro* assays revealed that tissue extracts from the lesion site of SCI are highly chemoattractive for USSC (see Fig. 4.2), suggesting that release of a chemoattractant is massively induced upon SC tissue damage which is in the healthy SC low abundant.

Recent reports documented that various potential chemoattractants are upregulated due to CNS disease including HGF (Honda et al., 1995; Kern et al., 2001; Tsuboi et al., 2002). The c-Met/HGF axis was shown to be involved in migration of various stem cell populations, including endothelial progenitor cells (Zhu et al., 2008), neural stem cells (Heese et al., 2005) and mesenchymal stem cells (Neuss et al., 2004). Of note, it has been demonstrated that USSC express the HGF receptor c-met evaluated by PCR and Western Blot analyses (Trapp et al., 2008).

The present study identified HGF as the responsible chemoattractant for USSC in SCI since neutralizing of HGF with a specific antibody (anti-HGF-AB) inhibited stimulation of USSC migration *in vitro*. This observation is in accordance with recent findings which revealed that HGF is up-regulated in lesion segments of spinal cord (Shimamura et al., 2007) and that HGF promotes migration of USSC in an animal model of focal cerebral ischemia (Trapp et al., 2008). The findings presented here underscore the important role of the HGF/c-met axis in regulating (stem) cell migration in CNS injuries. The reported migratory ability and the specific localization in the injury site further suggest that USSC may also be suitable therapeutic delivery vehicles for CNS disorders. In general, diseases caused by the lack of some crucial proteins led to the idea to restore the proteins by appropriate gene expression vectors. Delivery of specific therapeutically relevant molecules exactly released in the injury site might be achievable with USSC.

5.2 Transplanted USSC do not replace endogenous cells

ESC are quite promising for cell replacement approaches as they were shown to contribute to remyelination and functional recovery after transplantation into rodent SCI models (Sharp et al., 2010). Because ESC are highly proliferative, unrestricted in their development and sensitive to environmental cues for differentiation, teratoma formation cannot be excluded. Only few methods are described for pre-differentiation of highly purified progenitor populations of a desired cell type (Rossi and Keirstead, 2009). Interestingly, studies with NSC isolated from either fetal or adult material or differentiated from embryonic stem cells (for review see: Tetzlaff et al., 2010) described beneficial effects due to neural differentiation (Parr et al., 2007; Parr et al., 2008; Karimi-Abdolrazaee et al., 2006). However, the use of NSC harvested from human fetal tissue or derived from human embryonic stem cells raises severe ethical issues and technical difficulties. Furthermore, grafted NSC potentially promote neuropathic pain (Hofstetter et al., 2005; Macias et al., 2006) by lowering sensory thresholds to non-noxious stimuli (i.e. allodynia). Consequently, sources of adult stem cells were identified and investigated. In initial evaluations, beneficial effects observed after adult stem cell transplantation were assumed to result from transdifferentiation along diverse lineages, thereby generating new tissue. However, several studies have never provided compelling evidence in support of the notion that transdifferentiation is the basis of the therapeutic benefits which adult stem cells provide (English et al., 2006). Even in SCI research, numerous studies with adult stem cells from bone marrow were published reporting contradictory observations with respect to neural differentiation potential. Neural differentiation of only a few number of cells was reported by some groups (Akiyama et al., 2002; Cizkova et al. 2006, Park et al., 2010), whereas others could not detect neural lineage differentiation (Wu et al., 2003, Sheth et al., 2008). The ability of MSC derived from bone marrow to differentiate into neural cells still remains to be investigated (Lu and Tuszynski, 2005; Phinney and Prockop, 2007). Additionally, cell fusion of adult stem cells with host tissue cells could contribute to neural marker expression as fusion of bone marrow stem cells with neurons has been demonstrated *in vivo* (Alvarez-Dolado et al., 2003; Weimann et al., 2003). Moreover, *in vivo* differentiation of stem

cells derived from hUCB is controversially discussed (Brass 2006, English et al., 2006). Interestingly, supportive effects of transplanted hUBC stem cells in SCI have been described both in presence (Dasari et al., 2007; Kuh et al., 2005) or absence of neural differentiation (Saporta et al., 2003), thus indicating that neurogenesis of transplanted cells is unlikely to contribute to beneficial effects.

In the present thesis, there has been no evidence for USSC differentiation into neuronal or glial cells after transplantation into the injured rat spinal cord (see Fig. 4.3). Consequently, cell replacement by USSC as the mechanism responsible for the observed functional improvement is very unlikely. Instead, the present results encourage the idea to transplant cells which may promote regeneration via paracrine regulation rather than replacement mechanisms.

5.3 USSC are sufficient to promote functional recovery

Reports in the literature about functional outcome after transplantation of human somatic stem cells derived from bone marrow or umbilical cord blood are rather inconsistent (Himes et al., 2006, Cizkova et al., 2006; Neuhuber et al., 2005; Sheth et al., 2008). This could possibly be explained by the heterogeneity of the origin and insufficient characterization of the stem cell populations used, which mostly included the entire mononuclear cell fraction (for review: Tetzlaff et al., 2010). Furthermore, the functional outcomes reported must be cautiously interpreted, as most of the published results are solely based on a single functional test.

The present thesis revealed that USSC promote functional locomotor recovery after transplantation into a model of acute SCI (see chapter 4.4). In addition to the open field BBB locomotor score, which has certain limitations in the determination of forelimb-hindlimb coordination (Koopmans et al., 2005), the horizontal ladder walking test and the CatWalk gait analysis were performed. The horizontal ladder walking test was used to assess hindlimb placing and stepping, while the CatWalk gait analysis allowed us to determine interlimb coordination and individual paw parameters. At the beginning of the study (1 wpt), all injured animals showed equivalent deficits as indicated by similar open field BBB locomotor scores and

locomotor subscore values. With regard to the open field BBB subscore, the USSC grafted animals performed always better than the non-grafted control group reaching significance for the right hindlimb at 6 wpt (see Fig. 4.4). Furthermore, at 6 wpt and thereafter, USSC-transplanted animals made significantly less missteps with both hindlimbs in the horizontal ladder walking test (see Fig. 4.5). In addition, the assessment of static (relative paw placement, base of support, maximal contact area and maximal paw print area) as well as dynamic (swing speed, swing duration) gait parameters by means of CatWalk analysis revealed significant locomotor recovery for all gait parameters tested (see Fig. 4.6). The improved locomotor performances of USSC-transplanted rats was observed in all three behavioral tasks and remained until the end of the long-term (16 wpt) study, indicating a long-lasting functional benefit following transplantation of USSC into traumatic spinal cord injury.

Since neural differentiation has to be excluded (see chapter 4.3) thus indicating that the data described here regarding functional recovery after USSC-transplantation are independent of cell replacement, axonal regeneration and tissue preservation were analyzed.

5.4 USSC transplantation promotes axonal regeneration in an acute rat SCI model

Injury to the spinal cord interrupts ascending and descending fiber tracts of spinally projecting neurons which is associated with permanent motor and sensory deficits. SCI research focuses on the improvement of axon regeneration as regrowth of a low number of axons can lead to recovery of some respectable motor or sensory function (Fawcett, 1998). Various cell grafts have been shown to enhance axonal regeneration after SCI (Keirstead et al., 1999; Ramón-Cueto et al., 2000; Hofstetter et al., 2002; Pallini et al., 2005; Parr et al., 2008). In this thesis, it could be demonstrated that transplantation of USSC into the acutely injured spinal cord supports axonal regeneration. Three weeks after transplantation into the lesioned SC, large numbers of neurofilament-positive axons were found in close proximity to USSC, which were predominantly enriched at the injury site (see Fig. 4.7). To

investigate the potential of USSC to support axonal regeneration, anterograde axon tracing experiments were performed (see Fig. 4.8). Here, diffusion of the tracer substance into the surrounding spinal cord is a conceivable possibility for unspecific axon labeling, as the BDA-injection sites are in close proximity to the lesion area. In previous studies from our group, diffusion of the tracer substance has not been reported. Furthermore, as the number of BDA-positive fibers in the lesion site was very low in the presented experiments, it has been concluded that tracer diffusion did not occur in the current study. Only BDA-labeled structures which could clearly be identified as fibers were manually counted as the high background labeling in the lesion area of BDA-traced animals made it impossible to analyze the sections automatically. Quantification of BDA-traced fibers revealed that USSC transplantation enhances regenerative axon growth (see Fig. 4.9) which could account for the functional locomotor improvement found in the present thesis. Paracrine support as well as USSC-mediated contact guidance are possible mechanisms of axon growth promoting activity. It remains to be proven whether USSC-transplantation enhances regeneration of a specific fiber tract of the spinal cord.

5.5 USSC-secreted molecules lead to enhanced neurite growth

Exogenous delivery of neurotrophic factors has been proposed as therapeutic strategy for SCI repair and was tested in various pre-clinical studies which were shown to induce axonal regeneration and functional recovery (Tuszynski et al., 1996; Grill et al., 1997; Liu et al., 1999). The present thesis revealed that factors secreted by USSC could to be taken into account for the neurite growth promoting activity *in vivo* as incubation with USSC-conditioned medium (USSC-CM) led to enhanced neurite outgrowth of DRG explants and cortical neurons prepared from embryonic rats. DRG explants incubated with USSC-CM for 24h developed a significantly larger area of neurites compared to the controls (see Fig. 4.10). Additionally, neurite outgrowth and network formation was stimulated by USSC-CM (see Fig. 4.11) which is comparable in activity to astrocyte-conditioned medium (ACM). Recent reports evaluated that astrocytes release neurotrophic and neuritogenic factors that exert

neurite outgrowth and short time survival of primary neurons *in vitro* (Banker, 1980; Müller et al., 1984; Rudge et al., 1985). Several axon growth promoting molecules released by astrocytes were identified, such as NGF (Furukawa et al., 1986; Moretto et al., 1994), BDNF (Moretto et al., 1994), NT-3 (Rudge et al., 1992; Moretto et al., 1994) and FGF (Araujo and Cotman, 1992). Further analyses have to define whether USSC and astrocytes stimulate neurite outgrowth via same pathways since complete evaluation of USSC released factors is still lacking. At present, it is not clear whether USSC release the classical neurotrophic factors, e.g. NGF, BDNF or NT-3, *in vitro*. As shown previously (Kögler et al., 2005), native USSC release cytokines and growth factors with known neuroprotective and axon growth promoting functions. Comparison of the cytokine production between USSC and MSC derived from bone marrow revealed that USSC, in contrast to MSC, release certain growth factors at higher concentrations such as the vascular endothelial growth factor (VEGF) which is known to support neuronal survival (Sun et al., 2003; Tovar-y-Romo and Tapia, 2010), neurite outgrowth (Khaibullina et al., 2004) and tissue sparing in SCI (Kim et al., 2009). Furthermore, USSC release stromal derived factor-1 (SDF-1), which induces homing of NSC in the ischemic or injured brain (Imitola et al., 2004) and stimulates axonal sprouting in SCI (Opatz et al., 2009). Interestingly, USSC also release HGF, which is known to promote motorneuron survival and axonal regrowth, and is further considered as a guidance and survival factor during neural development (Ebens et al., 1996; Giacobini et al., 2007). HGF has been shown to promote axonal regrowth and functional recovery after SCI (Kitamura et al., 2007). However, the amount of endogenous USSC-derived HGF apparently is insufficient to disturb the external HGF gradient that originates from injured spinal cord tissue which attracts the USSC as shown *in vitro* (see Fig. 4.2). It should be noted that factors released by USSC might also trigger the expression of a cascade of secondary neurotrophic factors as shown for LIF, which is able to increase the expression of NT-3 after adult CNS trauma (Blesch et al., 1999). The different growth factors or combinations, therefore, are likely to participate in the beneficial effects observed after USSC transplantation into the injured spinal cord. Of note, growth factor expression profiling has been obtained *in vitro*. Since the SCI environment could affect the growth factor expression profile of USSC after transplantation, further

investigations are required. Moreover, besides a paracrine mechanism, support of axon growth by USSC-mediated contact guidance *in vivo* can not be excluded.

5.6 Enhanced tissue preservation after USSC tranplantation

Several studies have demonstrated that improved functional outcome is highly correlated with an increase in spared tissue (Little et al., 1988; Schucht et al., 2002; Ballermann and Fouad, 2006). Functional recovery may be the result of remyelination of spared fibers and adaptations in spared components of the CNS, termed plasticity. At present, stem cell transplantation appears to protect tissue as it could prevent irreversible loss of function, possibly by altering host environment to sufficiently enrich trophic support with a subsequent rescue of impaired host cells (Ourednik et al., 2002; Honma et al., 2006; Samandi et al., 2009). The present thesis demonstrated that factors secreted by USSC appear to be neuroprotective as they stimulate the survival of cortical neurons *in vitro* (see Fig. 4.11). As described before, USSC release various trophic factors, among others SDF-1, HGF and VEGF which are known to stimulate axonal growth. In recent reports, these factors were further described to exert neuroprotective effects suggesting that these factors contribute to the survival of cortical neurons described in the present thesis. For example, SDF-1 was found to be neuroprotective as it prevents cortical neurons and PC12 cells from apoptotic cell death *in vitro* (Shyu et al., 2008; Wang et al., 2010). Similarly, it has been described that HGF could enhance survival of hippocampal and mesencephalic neurons *in vitro* (Honda et al., 1995; Hamanoue et al., 1996; Miyazawa et al., 1998). Moreover, VEGF appears to have direct neuroprotective effects, as it increases the survival of cervical and dorsal root ganglion neurons (Sondell et al., 1999) and promotes the survival of mesencephalic neurons (Silverman et al., 1999) in culture. Whether the combination of the various trophic factors or one single factor secreted by USSC contribute to neuronal survival remains yet to be solved and is subject of future investigations.

To investigate whether USSC could enhance tissue preservation in SCI, the lesion size and spared tissue in USSC-transplanted and control rats were quantified

and compared as described elsewhere (Iannotti et al., 2010) with slight modifications. For quantitative evaluation, Image J software was used to determine the percentile area of the lesion. However, a stereological analysis using the Cavalieri method (Gundersen and Jensen, 1987) to estimate the volume of the lesion would be more precise and should be included in upcoming evaluations. The presented data revealed that grafting of USSC into traumatic SCI significantly reduces the lesion size and enhances the amount of spared tissue (see Fig. 4.12). Tissue sparing at the injury site upon USSC transplantation suggests to be directly related to the expeditious functional recovery. Whether USSC transplantation enhances white matter sparing which is known to be directly correlated with locomotor recovery in rats will be determined by future experiments. Thus, findings presented here confirm and exceed recent findings that somatic stem cells could reduce lesion volumes and prevent neural cell loss (Ankeny et al., 2004; Samdani et al., 2009; Gu et al., 2010; Osaka et al., 2010). Among other molecules, the secretion of SDF-1 by USSC could contribute to the enhanced tissue sparing after USSC-transplantation, as SDF-1 has been shown to reduce the volume of cerebral infarction due to up-regulation of anti-apoptotic proteins (Shyu et al., 2008). Additionally, HGF has been considered neuroprotective as exogenous HGF prevents neuronal cell death after forebrain ischemia and SCI (Miyazawa et al., 1998; Tsuzuki et al., 2001; Kitamura et al., 2007). Moreover, VEGF has been reported to enhance tissue sparing leading to improved functional recovery (Widenfalk et al., 2003; Kim et al., 2009).

5.7 Concluding remarks and further considerations

Data presented in this thesis demonstrate that transplantation of a well-defined somatic stem cell type from hUCB into an animal model of acute traumatic SCI leads to a significant long-lasting functional outcome as revealed by three different behavioral tests to investigate numerous task-specific locomotor parameters. The observed functional improvement correlates very well with reduced tissue loss or augmented tissue sparing and stimulation of regenerative axon growth. To accomplish the beneficial effects neither neural differentiation nor long-lasting

persistence of the grafted cells is required. The secretion of neurite outgrowth promoting factors *in vitro* further suggests a paracrine mechanism underlying the beneficial effects of USSC in SCI. Furthermore, USSC are able to invade the SC lesion site in a HGF-dependent manner, accumulate and survive for at least three weeks after transplantation.

In the present study, a dorsal hemisection model has been chosen, which is consistent and highly reproducible allowing precise investigations of axon regeneration. However, it would be interesting to investigate the effect of USSC transplantation on contusion injured rats, which is clinically more relevant as it represents the most common kind of injury in patients. Furthermore, it is currently unsolved whether USSC transplantation leads to more pronounced improvement than MSC from human umbilical cord blood or adult bone marrow. These aspects will be taken into consideration for future projects where the functional outcome following grafting of USSC and MSC from both cord blood (CB-MSC) and bone marrow (BM-MSC) sources into the lesioned rat spinal cord will be compared in acute SCI models, but also in subacute and chronic lesion models. Additionally, further analyses are required to investigate whether the beneficial effects observed after USSC transplantation are due to released trophic molecules and/or cell-bound factors. Application of USSC-CM by intrathecal infusion via osmotic mini-pumps into the lesioned spinal cord might be one possible experimental approach to investigate the potential role of USSC secreted molecules. Additionally, it will be of particular interest to examine in future whether USSC contribute to spinal plasticity as compensatory sprouting of injured corticospinal axons has been shown to contribute to limited recovery due to an increase in connectivity (Hill et al., 2001; Rainetau and Schwab, 2001; Weidner et al., 2001).

Given the highly supportive functional characteristics without adverse effects in SCI, the possibility to produce USSC in virtually unlimited quantities at GMP-grade without ethical concerns, USSC transplantation appears as an effective strategy to promote functional regeneration after SCI.

6. References

Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, Small JE, Herrlinger U, Ourednik V, Black PM, Breakefield XO, Snyder EY (2000) Neural stem cells display extensive tropism for pathology in adult brain: Evidence from intracranial gliomas. Proc Natl Acad Sci USA 97:12846–12851.

Afshari FT, Kappagantula S, Fawcett JW (2009) Extrinsic and intrinsic factors controlling axonal regeneration after spinal cord injury. Expert Rev Mol Med 11:e37.

Akiyama Y, Radtke C, Kocsis JD (2002) Remyelination of rat spinal cord by transplantation of identified bone marrow stromal cells. J Neurosci 22:6623-6630.

Aktas M, Buchheiser A, Houben A, Reimann V, Radke T, Jeltsch K, Maier P, Zeller WJ, Kögler G (2010) Good manufacturing practice-grade production of unrestricted somatic stem cell from fresh cord blood. Cytotherapy 12:338-348.

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

Ankeny DP, McTigue DM, Jakeman LG (2004) Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats. Exp Neurol 190:17-31.

Ara T, Tokoyoda K, Sugiyama T, Egawa T, Kawabata K, Nagasawa T (2003) Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. Immunity 19:257-267.

Araujo DM, Cotman CW (1992) Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. J Neurosci 5:1668-1678.

Arcese W, Rocha V, Labopin M, Sanz G, Iori AP, de Lima M, Sirvent A, Busca A, Asano S, Ionescu I, Wernet P, Gluckman E; Eurocord-Netcord Transplant group (2006) Unrelated cord blood transplants in adults with hematologic malignancies. Haematologica 91:223-230.

Azari MF, Profyris C, Karnezis T, Bernard CC, Small DH, Cheema SS, Ozturk E, Hatzinisiriou I, Petratos S (2006) Leukemia inhibitory factor arrests oligodendrocyte death and demyelination in spinal cord injury. J Neuropathol Exp Neurol 65:914-929.

Ballermann M, Fouad K (2006) Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. Eur J Neurosci 8:1988-1996.

Balordi F, Fishell G (2007) Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. J Neurosci 27:5936-5947.

Bamber NI, Li H, Lu X, Oudega M, Aebischer P, Xu XM (2001) Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seeded mini-channels. Eur J Neurosci 13:257-268.

Banker GA (1980) Trophic interactions between astroglial cells and hippocampal neurons in culture. Science 209:809-810.

Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, McGlave PB, Miller JS, Verfaillie CM, Wagner JE (2005) Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. Blood 105:1343-1347.

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449:1003-1007.

Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 12:1–21.

Beattie MS, Bresnahan JC, Komon J, Tovar CA, Van Meter M, Anderson DK, Faden AI, Hsu CY, Noble LJ, Salzman S, Young W (1997) Endogenous repair after spinal cord contusion injuries in the rat. Exp Neurol 148:453-463.

Becerra JL, Puckett WR, Hiester ED, Quencer RM, Marcillo AE, Post MJ, Bunge RP (1995) MR-pathologic comparisons of wallerian degeneration in spinal cord injury. AJNR Am J Neuroradiol 16:125-133.

Benedetti S, Pirola B, Pollo B, Magrassi L, Bruzzone MG, Rigamonti D, Galli R, Selleri S, Di Meco F, De Fraja C, Vescovi A, Cattaneo E, Finocchiaro G (2000) Gene therapy of experimental brain tumors using neural progenitor cells. Nat Med 4:447-450.

Benfey M, Aguayo AJ (1982) Extensive elongation of axons from rat brain into peripheral nerve grafts. Nature 296:150–152.

Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, Mancardi G, Uccelli A (2007) Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells 7:1753-60.

Betschinger J, Knoblich JA (2004) Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol 14:R674–R685.

Betschinger J, Mechtler K, Knoblich JA (2006) Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell 124:1241–1253.

Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF (2003). Met, metastasis, motility and more. Nat Rev Mol Cell Biol 4:915-925.

Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C (1995) Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 376:768-771.

Blesch A, Uy HS, Grill RJ, Cheng JG, Patterson PH, Tuszynski MH (1999) LIF augments corticospinal axon growth and neurotrophin expression after adult CNS injury. J Neurosci 19:3556–3566.

Blesch A, Tuszynski MH (2003) Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination. J Comp Neurol 467:403-417.

Bottenstein JE, GH Sato (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc Natl Acad Sci USA 76:514-517.

Bradbury EJ, McMahon SB (2006) Spinal cord repair strategies: why do they work? Nat Rev Neurosci 7:644-653.

Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416:636-640.

Brass LM (2006) Bone marrow for brain repair? Exp Neurol 199:16-19.

Brock JH, Rosenzweig ES, Blesch A, Moseanko R, Havton LA, Edgerton VR, Tuszynski MH (2010) Local and remote growth factor effects after primate spinal cord injury. J Neurosci 29:9728-9737.

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425:841-846.

Cao QL, Zhang YP, Howard RM, Walters WM, Tsoulfas P, and Whittemore SR (2001). Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp Neurol 167:48-58.

Cao QL, Howard RM, Dennison JB, Whittemore SR (2002). Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. Exp Neurol 177:349-359.

Caroni P, Schwab ME (1988) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1:85-96.

Chau CH, Shum DK, Li H, Pei J, Lui YY, Wirthlin L, Chan YS, XuXM (2004) Chondroitinase ABC enhances axonal regrowth through Schwann cells seeded guidance channels after spinal cord injury. FASEB J 18:194–196.

Chaudhry V, Glass JD, Griffin JW (1992) Wallerian degeneration in peripheral nerve disease. Neurol Clin 3:613-627.

Chernykh ER, Stupak VV, Muradov GM, Sizikov MY, Shevela EY, Leplina OY, Tikhonova MA, Kulagin AD, Lisukov IA, Ostanin AA, Kozlov VA (2007) Application of autologous bone marrow stem cells in the therapy of spinal cord injury patients. Bull Exp Biol Med 4:543-547.

Cho SR, Yang MS, Yim SH, Park JH, Lee JE, Eom YW, Jang IK, Kim HE, Park JS, Kim HO, Lee BH, Park CI, Kim YJ (2008) Neurally induced umbilical cord blood cells modestly repair injured spinal cords. Neuroreport 13:1259-1263.

Chung Y, Klimanskaya I, Becker S, Marh J, Lu SJ, Johnson J, Meisner L, Lanza R (2006) Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. Nature 439:216-219.

Cizkova D, Rosocha J, Vanicky I, Jergova S, Cizek M (2006) Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. Cell Mol Neurobiol 26:1167-1180.

Claudinot S, Nicolas M, Oshima H, Rochat A, Barrandon Y (2005) Long-term renewal of hair follicles from clonogenic multipotent stem cells. Proc Natl Acad Sci USA 102:14677-14682.

Coltella N, Manara MC, Cerisano V, Trusolino L, Di Renzo MF, Scotlandi K, Ferracini R (2003) Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. FASEB J 17(9):1162-1164.

Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS (2001) Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. J Neurosci 21:9334–9344.

Cristante AF, Barros-Filho TEP, Tatsui N, Mendrone A, Caldas JG, Camargo A, Alexandre A, Teixeira WGJ, Oliveira RP, Marcon MR (2009) Stem cells in the treatment of chronic spinal cord injury: evaluation of somatosensitive evoked potentials in 39 patients. Spinal Cord 47:733–738.

Dasari VR, Spomar DG, Gondi CS, Sloffer CA, Saving KL, Gujrati M, Rao JS, Dinh DH (2007) Axonal remyelination by cord blood stem cells after spinal cord injury. J Neurotrauma 24:391-410.

Dasari VR, Spomar DG, Li L, Gujrati M, Rao JS, Dinh DH (2008) Umbilical cord blood stem cell mediated downregulation of fas improves functional recovery of rats after spinal cord injury. Neurochem Res 33:134-149.

Dasari VR, Veeravalli KK, Tsung AJ, Gondi CS, Gujrati M, Dinh DH, Rao JS (2009) Neuronal apoptosis is inhibited by cord blood stem cells after spinal cord injury. J Neurotrauma 11:2057-2069.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system “bridges” after central nervous system injury in adult rats. Science 214:931–933.

de Lima M, McMannis J, Gee A, Komanduri K, Couriel D, Andersson BS, Hosing C, Khouri I, Jones R, Champlin R, Karandish S, Sadeghi T, Peled T, Grynspan F, Daniely Y, Nagler A, Shpall EJ (2008) Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. Bone Marrow Transplant 41:771-778.

Deda H, Mc İnci, Kürekçi AE, Kayıhan K, Özgün E, Üstünsoy GE, Kocabay S (2008) Treatment of chronic spinal cord injured patients with autologous bone marrow-derived hematopoietic stem cell transplantation: 1-year follow-up. Spinal Cord 10:565-574.

Deguchi K, Yagi H, Inada M, Yoshizaki K, Kishimoto T, Komori T (1999) Excessive extramedullary hematopoiesis in Cbfa1-deficient mice with a congenital lack of bone marrow. Biochem Biophys Res Commun 255:352-359.

Delehedde M, Sergeant N, Lyon M, Rudland PS, Fernig DG (2001) Hepatocyte growth factor/scatter factor stimulates migration of rat mammary fibroblasts through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways. Eur J Biochem 268:4423-4429.

Di Giovanni S (2009) Molecular targets for axon regeneration: focus on the intrinsic pathways. Expert Opin Ther Targets 13:1387-1398.

Dimou L, Schnell L, Montani L, Duncan C, Simonen M, Schneider R, Liebscher T, Gullo M, Schwab ME (2006) Nogo-A-deficient mice reveal strain-dependent differences in axonal regeneration. J Neurosci 26:5591–5603.

Dou CL, Levine JM (1994) Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. J Neurosci 14:7616–7628.

Ducy P, Schinke T, Karsenty G (2000) The osteoblast: a sophisticated fibroblast under central surveillance. Science 289:1501-1504.

Ebens A, Brose K, Leonardo ED, Hanson MG Jr, Bladt F, Birchmeier C, Barres BA, Tessier-Lavigne M (1996) Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron 6:1157-1172.

English D, Klasko SK, Sanberg PR (2006) Elusive mechanisms of “stem cell”-mediated repair of cerebral damage. Exp Neurol 199:10-15.

Enomoto M, Shinomiya K, Okabe S (2003) Migration and differentiation of neural progenitor cells from two different regions of embryonic central nervous system after transplantation into the intact spinal cord. Eur J Neurosci 6:1223-1232.

Enzmann GU, Benton RL, Talbott JF, Cao Q, Whittemore SR (2006) Functional considerations of stem cell transplantation therapy for spinal cord repair. J Neurotrauma 3-4:479-495.

Erdö F, Bührle C, Blunk J, Hoehn M, Xia Y, Fleischmann B, Föcking M, Küstermann E, Kolossov E, Hescheler J, Hossmann KA, Trapp T (2003) Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. J Cereb Blood Flow Metab 23:780-785.

Erices A, Conget P, Minguell JJ (2000) Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 109:235.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 24:2143-2155.

Fawcett JW (1998) Spinal cord repair: from experimental models to human application. Spinal Cord 36:811-817.

Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377-391.

Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4:703-713.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD, Pasquale-Styles M, Dietrich WD, Weaver LC (2006) The cellular inflammatory response in human spinal cords after injury. Brain 129:3249-3269.

Fouad K, Schnell L, Bunge MB, Schwab ME, Liebscher T, Pearse DD (2005) Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. J Neurosci 5:1169-1178.

Franklin RJ, Bayley SA, Blakemore WF (1996) Transplanted CG4 cells (an oligodendrocyte progenitor cell line) survive, migrate, and contribute to repair of areas of demyelination in X-irradiated and damaged spinal cord but not in normal spinal cord. Exp Neurol 137:263-276.

Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K (1986) Synthesis and secretion of nerve growth factor by mouse astroglial cells in culture. Biochem Biophys Res Commun 136:57-63.

Furuya T, Hashimoto M, Koda M, Okawa A, Murata A, Takahashi K, Yamashita T, Yamazaki M (2009) Treatment of rat spinal cord injury with a Rho-kinase inhibitor and bone marrow stromal cell transplantation. Brain Res 1295:192-202.

Gang EJ, Hong SH, Jeong JA, Hwang SH, Kim SW, Yang IH, Ahn C, Han H, Kim H (2004) In vitro mesengenic potential of human umbilical cord blood-derived mesenchymal stem cells. Biochem Biophys Res Commun 321:102-108.

Garcia-Marques J, De Carlos JA, Greer CA, Lopez-Mascaraque L (2010) Different astroglia permissivity controls the migration of olfactory bulb interneuron precursors. Glia 58:218-230.

Geffner LF, Santacruz P, Izurieta M, Flor L, Maldonado B, Auad AH, Montenegro X, Gonzalez R, Silva F (2008) Administration of autologous bone marrow stem cells into spinal cord injury patients via multiple routes is safe and improves their quality of life: comprehensive case studies. Cell Transplant 17:1277-1293.

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

Giacobini P, Messina A, Wray S, Giampietro C, Crepaldi T, Carmeliet P, Fasolo A (2007) Hepatocyte growth factor acts as a motogen and guidance signal for gonadotropin hormone-releasing hormone-1 neuronal migration. J Neurosci 27:431-445.

Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P, et al (1989) Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 321:1174-1178.

Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA (2001) Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant 7:581-588.

Greschat S, Schira J, Küry P, Rosenbaum C, de Souza Silva MA, Kögler G, Wernet P, Müller HW (2008) Unrestricted somatic stem cells from human umbilical cord blood can be differentiated into neurons with a dopaminergic phenotype. Stem Cells Dev 17:221-232.

Grill R, Murai K, Blesch A, Gage FH, Tuszynski MH (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. J Neurosci 14:5560-5572.

Grill RJ, Blesch A, Tuszynski MH (1997) Robust growth of chronically injured spinal cord axons induced by grafts of genetically modified NGF-secreting cells. Exp Neurol 148:444-452.

Grimpe B, Silver J (2002) The extracellular matrix in axon regeneration. Prog Brain Res 137:333-349.

Gu W, Zhang F, Xue Q, Ma Z, Lu P, Yu B (2010) Transplantation of bone marrow mesenchymal stem cells reduces lesion volume and induces axonal regrowth of injured spinal cord. Neuropathology 30:205-217.

Gundersen HJ, Jensen EB (1987) The efficiency of systematic sampling in stereology and its prediction. J Microsc 147:229-263.

Hamanoue M, Takemoto N, Matsumoto K, Nakamura T, Nakajima K, Kohsaka S (1996) Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J Neurosci Res 43:554-564.

Hamers FP, Lankhorst AJ, Van Laar TJ, Veldhuis WB, Gispen WH (2001) Automated quantitative gait analysis during overground locomotion in the rat: Its application to spinal cord contusion and transection injuries. J Neurotrauma 18: 187–201.

Hamers FP, Koopmans GC, Joosten EA (2006) CatWalk-assisted gait analysis in the assessment of spinal cord injury. J Neurotrauma 23:537-548.

Hattori K, Heissig B, Tashiro K, Honjo T, Tateno M, Shieh JH, Hackett NR, Quitoriano MS, Crystal RG, Rafii S, Moore MA (2001) Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood 97:3354-3360.

He F, Wu LX, Shu KX, Liu FY, Yang LJ, Zhou X, Zhang Y, Huang BS, Huang D, Deng XL (2007) HGF protects cultured cortical neurons against hypoxia/reoxygenation induced cell injury via ERK1/2 and PI-3K/Akt pathways. Colloids Surf B Biointerfaces 61:290-297.

Heese O, Disko A, Zirkel D, Westphal M, Lamszus K (2005) Neural stem cell migration toward gliomas in vitro. Neuro Oncol 7:476-484.

Hermanns S, Reiprich P, Müller HW (2001) A reliable method to reduce collagen scar formation in the lesioned rat spinal cord. J Neurosci Methods 110:141-146.

Hill CE, Beattie MS, Bresnahan JC (2001) Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. Exp Neurol 171:153-169.

Hill WD, Hess DC, Martin-Studdard A, Carothers JJ, Zheng J, Hale D, Maeda M, Fagan SC, Carroll JE, Conway SJ (2004) SDF-1 (CXCL12) is upregulated in the ischemic penumbra following stroke: association with bone marrow cell homing to injury. J Neuropathol Exp Neurol 63:84-96.

Himes BT, Neuhuber B, Coleman C, Kushner R, Swanger SA, Kopen GC, Wagner J, Shumsky JS, Fischer I (2006) Recovery of function following grafting of human bone marrow-derived stromal cells into the injured spinal cord. Neurorehabil Neural Repair 20:278–296.

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

Hofstetter CP, Holmstrom NA, Lilja JA, Schweinhardt P, Hao J, Spenger C, Wiesenfeld-Hallin Z, Kurpad SN, Frisen J, Olson L (2005) Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat Neurosci 8:346-353.

Honda S, Kagoshima M, Wanaka A, Tohyama M, Matsumoto K, Nakamura T (1995) Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor. Brain Res Mol Brain Res 32:197-210.

http://www.colorado.edu/intphys/Class/IPHY3430-200/image/09-7.jpg

http://www.geron.com

http://www.rickhansenregistry.org

Hsu JY, Xu XM (2005) Early profiles of axonal growth and astroglial response after spinal cord hemisection and implantation of Schwann cell-seeded guidance channels in adult rats. J Neurosci Res 82:472-483.

Hurtado-Chong A, Yusta-Boyo MJ, Vergano-Vera E, Bulfone A, de Pablo F, Vicario-Abejon C (2009) IGF-I promotes neuronal migration and positioning in the olfactory bulb and the exit of neuroblasts from the subventricular zone. Eur J Neurosci 30:742-755.

Iannotti CA, Clark M, Horn KP, van Rooijen N, Silver J, Steinmetz MP (2010) A combination immunomodulatory treatment promotes neuroprotection and locomotor recovery after contusion SCI. Exp Neurol 2010 [Epub ahead of print].

Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, Snyder EY, Khoury SJ (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci USA 101:18117-18122.

Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, Cotsarelis G (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nat Med 12:1351-1354.

Itoh T, Satou T, Ishida H, Nishida S, Tsubaki M, Hashimoto S, Ito H (2009) The relationship between SDF-1alpha/CXCR4 and neural stem cells appearing in damaged area after traumatic brain injury in rats. Neurol Res 31:90-102.

Iwaniuk KM, Schira J, Weinhold S, Jung M, Adjaye J, Müller HW, Wernet P, Trompeter HI (2010) Network-like Impact of MicroRNAs on Neuronal Lineage Differentiation of Unrestricted Somatic Stem Cells from Human Cord Blood (USSC). Stem Cells Dev [Epub ahead of print].

Jakeman LB, Wei P, Guan Z, Stokes BT (1998) Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. Exp Neurol 154:170-184.

Jansen BJ, Gilissen C, Roelofs H, Schaap-Oziemlak A, Veltman JA, Raymakers RA, Jansen JH, Kögler G, Figdor CG, Torensma R, Adema GJ (2010) Functional differences between mesenchymal stem cell populations are reflected by their transcriptome. Stem Cells Dev 19:481-490.

Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:421-426.

Jin KL, Mao XO, Greenberg DA (2000) Vascular endothelial growth factor: direct neuroprotective effect in *in vitro* ischemia. Proc Natl Acad Sci USA 97:10242-10247.

Kakulas BA (1999) A review of the neuropathology of human spinal cord injury with emphasis on special features. J Spinal Cord Med 22:119–124.

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

Kasemeier-Kulesa JC, McLennan R, Romine MH, Kulesa PM, Lefcort F (2010) CXCR4 controls ventral migration of sympathetic precursor cells. J Neurosci 39:13078-13088.

Keirstead HS, Morgan SV, Wilby MJ, Fawcett JW (1999) Enhanced axonal regeneration following combined demyelination plus schwann cell transplantation therapy in the injured adult spinal cord. Exp Neurol 159:225-236.

Kern MA, Friese M, Grundstrom E, Korhonen L, Wallin A, Aquilonius SM, Askmark H, Schirmacher P, Lindholm D (2001) Amyotrophic lateral sclerosis: evidence for intact hepatocyte growth factor/met signalling axis. Cytokine 15:315-319.

Kerr BJ, Patterson PH (2005) Leukemia inhibitory factor promotes oligodendrocyte survival after spinal cord injury. Glia 51:73-79.

Khaibullina AA, Rosenstein JM, Krum JM (2004) Vascular endothelial growth factor promotes neurite maturation in primary CNS neuronal cultures. Brain Res Dev Brain Res 148:59-68.

Khwaja A, Lehmann K, Marte BM, Downward J (1998) Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. J Biol Chem 273:18793-18801.

Kim HM, Hwang DH, Lee JE, Kim SU, Kim BG (2009) *Ex Vivo* VEGF delivery by neural stem cells enhances proliferation of glial progenitors, angiogenesis, and tissue sparing after spinal cord injury. PLoS ONE 4:e4987.

Kitamura K, Iwanami A, Nakamura M, Yamane J, Watanabe K, Suzuki Y, Miyazawa D, Shibata S, Funakoshi H, Miyatake S, Coffin RS, Nakamura T, Toyama Y, Okano H (2007) Hepatocyte growth factor promotes endogenous repair and functional recovery after spinal cord injury. J Neurosci Res 85:2332-2342.

Klapka N, Hermanns S, Straten G, Masanneck C, Duis S, Hamers FP, Müller D, Zuschratter W, Müller HW (2005) Suppression of fibrous scarring in spinal cord injury of rat promotes long-distance regeneration of corticospinal tract axons, rescue of primary motoneurons in somatosensory cortex and significant functional recovery. Eur J Neurosci 12:3047-3058.

Klapka N, Müller HW (2006) Collagen matrix in spinal cord injury. J Neurotrauma 23:422-435.

Kluth SM, Buchheiser A, Houben AP, Geyh S, Krenz T, Radke TF, Wiek C, Hanenberg H, Reinecke P, Wernet P, Kögler G (2010) DLK-1 as a marker to distinguish unrestricted somatic stem cells and mesenchymal stromal cells in cord blood. Stem Cells Dev 10:1471-1483.

Knoblich, JA (2008) Mechanisms of asymmetric cell division. Cell 132:583-597.

Kodama A, Matozaki T, Fukuhara A, Kikyo M, Ichihashi M, Takai Y (2000) Involvement of an SHP-2-Rho small G protein pathway in hepatocyte growth factor/scatter factor-induced cell scattering. Mol Biol Cell 11:2565-2575.

Kögler G, Sensken S, Airey JA, Trapp T, Müschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Müller HW, Zanjani E, Wernet P (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123-135.

Kögler G, Radke TF, Lefort A, Sensken S, Fischer J, Sorg RV, Wernet P (2005) Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. Exp Hematol 33:573-583.

Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, Shizuru JA, Weissman IL (2003) Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol 21:759-806.

Koopmans GC, Deumens, Honig WM, Hamers FP, Steinbusch HW, Joosten EA (2005) The assessment of locomotor function in spinal cord injured rats: the importance of objective analysis of coordination. J Neurotrauma 22:214-225.

Kuh SU, Cho YE, Yoon DH, Kim KN, Ha Y (2005) Functional recovery after human umbilical cord blood cells transplantation with brain-derived neurotrophic factor into the spinal cord injured rat. Acta Neurochir (Wien) 147:985-992.

Lee HJ, Lee JK, Lee H, Carter JE, Chang JW, Oh W, Yang YS, Suh JG, Lee BH, Jin HK, Bae JS (2010) Human umbilical cord blood-derived mesenchymal stem cells improve neuropathology and cognitive impairment in an Alzheimer's disease mouse model through modulation of neuroinflammation. Neurobiol Aging. [Epub ahead of print]

Lee J, Kuroda S, Shichinohe H, Ikeda J, Seki T, Hida K, Tada M, Sawada K, Iwasaki Y (2003) Migration and differentiation of nuclear fluorescence-labeled bone marrow stromal cells after transplantation into cerebral infarct and spinal cord injury in mice. Neuropathology 3:169-180.

Lee JK, Chow R, Xie F, Chow SY, Tolentino KE, Zheng B (2010) Combined genetic attenuation of myelin and semaphorin-mediated growth inhibition is insufficient to promote serotonergic axon regeneration. J Neurosci 32:10899-10904.

Lee TT, Green BA, Dietrich WD, Yezierski RP (1999) Neuroprotective effects of basic fibroblast growth factor following spinal cord contusion injury in the rat. J Neurotrauma 5:347-356.

Lekakis L, Giralt S, Couriel D, Shpall EJ, Hosing C, Khouri IF, Anderlini P, Korbling M, Martin T, Champlin RE, de Lima M (2006) Phase II study of unrelated cord blood transplantation for adults with high-risk hematologic malignancies. Bone Marrow Transplant 38:421-426.

Levine JM (1994) Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. J Neurosci 8:4716-4730.

Liebscher T, Schnell L, Schnell D, Scholl J, Schneider R, Gullo M, Fouad K, Mir A, Rausch M, Kindler D, Hamers FP, Schwab ME (2005) Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. Ann Neurol 58:706–719.

Liedtke S, Buchheiser A, Bosch J, Bosse F, Kruse F, Zhao X, Santourlidis S, Kögler G (2010) The HOX Code as a "biological fingerprint" to distinguish functionally distinct stem cell populations derived from cord blood. Stem Cell Res 5:40-50.

Little JW, Harris RM, Sohlberg RC (1988) Locomotor recovery following subtotal spinal cord lesions in a rat model. Neurosci Lett 87:189-194.

Liu Y, Kim D, Himes BT, Chow SY, Schallert T, Murray M, Tessler A, Fischer I (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. J Neurosci 11:4370-4387.

Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145-1148.

Lois C, Garcia-Verdugo JM, Alvarez-Buylla A (1996) Chain migration of neuronal precursors. Science 271:978-981.

Lu P, Jones LL, Tuszynski MH (2007) Axon regeneration through scars and into sites of chronic spinal cord injury. Exp Neurol 203:8–21.

Luo J, Zhang HT, Jiang XD, Xue S, Ke YQ (2009) Combination of bone marrow stromal cell transplantation with mobilization by granulocyte-colony stimulating factor promotes functional recovery after spinal cord transection. Acta Neurochir (Wien) 11:1483-1492.

Lyman SD, Jacobsen SE (1998) c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood 91:1101–1134.

Ma PC, Tretiakova MS, Nallasura V, Jagadeeswaran R, Husain AN, Salgia R (2007) Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion. Br J Cancer 3:368-377.

Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA (1998) Impaired Blymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci USA 95:9448-9453.

Macias MY, Syring MB, Pizzi MA, Crowe MJ, Alexanian AR, Kurpad SN (2006) Pain with no gain: allodynia following neural stem cell transplantation in spinal cord injury. Exp Neurol 201:335-348.

Magavi SS, Mitchell BD, Szentirmai O, Carter BS, Macklis JD (2005) Adult-born and pre-existing olfactory granule neurons undergo distinct experience-dependent modifications of their olfactory responses in vivo. J Neurosci 25:10729-10739.

Markus A, Zhong J, Snider WD (2002) Raf and akt mediate distinct aspects of sensory axon growth. Neuron 35:65-76.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.

McTigue DM, Horner PJ, Stokes BT, Gage FH (1998) Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci 14:5354-5365.

Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829-834.

Merkler D, Metz GA, Raineteau O, Dietz V, Schwab ME, Fouad K (2001) Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. J Neurosci 21:3665–3673.

Metz GA, Merkler D, Dietz V, Schwab ME, Fouad K (2000) Efficient testing of motor function in spinal cord injured rats. Brain Res 883: 165–177.

Metz GA, Whishaw IQ (2002) Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate foreand hind limb stepping, placing, and co-ordination. J Neurosci Meth 115:169–179.

Miyazawa K, Shimomura T, Naka D, Kitamura N (1994) Proteolytic activation of hepatocyte growth factor in response to tissue injury. J Biol Chem 269:8966–8970.

Miyazawa T, Matsumoto K, Ohmichi H, Katoh H, Yamashima T, Nakamura T (1998) Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: a novel neurotrophic factor. J Cereb Blood Flow Metab 4:345-348.

Moon LD, Asher RA, Rhodes KE, Fawcett JW (2001) Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. Nature Neurosci 4:465–466.

Moretto G, Xu RY, Walker DG, Kim SU (1994) Co-expression of mRNA for neurotrophic factors in human neurons and glial cells in culture. J Neuropathol Exp Neurol 53:78-85.

Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G (2004) Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 22:411-417.

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.

Müller FJ, Snyder EY, Loring JF (2006) Gene therapy: can neural stem cells deliver? Nat Rev Neurosci 1:75-84.

Müller HW, Beckh S, Seifert W (1984) Neurotrophic factor for central neurons. Proc Natl Acad Sci USA 81:1248-1252.

Mullner A, Gonzenbach RR, Weinmann O, Schnell L, Liebscher T, Schwab ME (2008) Lamina-specific restoration of serotonergic projections after Nogo-A antibody treatment of spinal cord injury in rats. Eur J Neurosci 27:326–333.

Murphy P, Topilko P, Schneider-Maunoury S, Seitanidou T, Baron-Van Evercooren A, Charnay P (1996) The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. Development 122:2847–2857.

Nelson RD, Quie PG, Simmons RL (1975) Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J Immunol 115:1650-1656.

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

Neuss S, Becher E, Wöltje M, Tietze L, Jahnen-Dechent W (2004) Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. Stem Cells 22:405-414.

Opatz J, Küry P, Schiwy N, Järve A, Estrada V, Brazda N, Bosse F, Müller HW (2009) SDF-1 stimulates neurite growth on inhibitory CNS myelin. Mol Cell Neurosci 40:293-300.

Osaka M, Honmou O, Murakami T, Nonaka T, Houkin K, Hamada H, Kocsis JD (2010) Intravenous administration of mesenchymal stem cells derived from bone marrow after contusive spinal cord injury improves functional outcome. Brain Res 1343:226-235.

Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242-245.

Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY (2002) Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotechnol 11:1103-1110.

Pal R, Venkataramana NK, Bansal A, Balaraju S, Jan M, Chandra R, Dixit A, Rauthan A, Murgod U, Totey S (2009) Ex vivo-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. Cytotherapy 11:897-911.

Pallini R, Vitiani LR, Bez A, Casalbore P, Facchiano F, Di Giorgi Gerevini V, Falchetti ML, Fernandez E, Maira G, Peschle C, Parati E (2005) Homologous transplantation of neural stem cells to the injured spinal cord of mice. Neurosurgery 57:1014-1025.

Park HW, Lim MJ, Jung H, Lee SP, Paik KS, Chang MS (2010) Human mesenchymal stem cell-derived Schwann cell-like cells exhibit neurotrophic effects, via distinct growth factor production, in a model of spinal cord injury. Glia 58:1118-1132.

Park K, Luo JM, Hisheh S, Harvey AR, Cui Q (2004) Cellular mechanisms associated with spontaneous and ciliary neurotrophic factor-cAMP-induced survival and axonal regeneration of adult retinal ganglion cells. J Neurosci 24:10806-10815.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322:963-966.

Park KK, Liu K, Hu Y, Kanter JL, He Z (2010) PTEN/mTOR and axon regeneration. Exp Neurol 223:45-50.

Parr AM, Kulbatski I, Tator CH (2007) Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury. J Neurotrauma 24:835-845.

Parr AM, Kulbatski I, Zahir T, Wang X, Yue C, Keating A, Tator CH (2008) Transplanted adult spinal cord-derived neural stem/progenitor cells promote early functional recovery after rat spinal cord injury. Neuroscience 155:760-770.

Pearse DD, Marcillo AE, Oudega M, Lynch MP, Wood PM, Bunge MB (2004) Transplantation of Schwann cells and olfactory ensheathing glia after spinal cord injury: can pretreatment with methylprednisolone and interleukin-10 enhance recovery? J Neurotrauma 21:1223–1239.

Pecora AL, Stiff P, Jennis A, Goldberg S, Rosenbluth R, Price P, Goltry KL, Douville J, Armstrong RD, Smith AK, Preti RA (2000) Prompt and durable engraftment in two older adult patients with high risk chronic myelogenous leukemia (CML) using ex vivo expanded and unmanipulated unrelated umbilical cord blood. Bone Marrow Transplant 25:797-799.

Phinney DG, Prockop DJ (2007) Concise review: mesenchymal stem/ multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views. Stem Cells 25:2896–2902.

Pimentel-Coelho PM, Magalhães ES, Lopes LM, deAzevedo LC, Santiago MF, Mendez-Otero R (2010). Human cord blood transplantation in a neonatal rat model of hypoxic-ischemic brain damage: functional outcome related to neuroprotection in the striatum. Stem Cells Dev 3:351-358.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

Pollack SJ, Harper SJ (2002) Trk Neurotrophin Receptor Activators. Drug News Perspect 5:268-277.

Powell EM, Mars WM, Levitt P (2001) Hepatocyte growth factor/scatter factor is a motogen for interneurons migrating from the ventral to dorsal telencephalon. Neuron 1:79-89.

Raineteau O, Schwab ME (2001) Plasticity of motor systems after incomplete spinal cord injury. Nature Rev Neurosci 2:263–273.

Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, Hristova M, Nateri AS, Makwana M, Ls RS, Wolfer DP, Lipp HP, Aguzzi A, Wagner EF, Behrens A (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43:57–67.

Rajan P, Stewart CL, Fink JS (1995) LIF-mediated activation of STAT proteins after neuronal injury in vivo. Neuroreport 6:2240-2244.

Ramón-Cueto A, Cordero MI, Santos-Benito FF, Avila J (2000) Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 2:425-435.

Rando TA (2006) Stem cells, ageing and the quest for immortality. Nature 441:1080–1086.

Reichert WM (2008): Indwelling Neural Implants. Strategies for Contending with the *In Vivo* Environment. Frontiers in Neuroengineering. ISBN-13: 978-0-8493-9362-4

Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer and cancer stem cells. Nature 414:105–111.

Rezaie P, Trillo-Pazos G, Everall IP, Male DK (2002) Expression of beta-chemokines and chemokine receptors in human fetal astrocyte and microglial co-cultures: potential role of chemokines in the developing CNS. Glia 37:64-75.

Robin AM, Zhang ZG, Wang L, Zhang RL, Katakowski M, Zhang L, Wang Y, Zhang C, Chopp M (2006) Stromal cell-derived factor 1alpha mediates neural progenitor cell motility after focal cerebral ischemia. J Cereb Blood Flow Metab 26:125-134.

Rosenkranz K, Kumbruch S, Lebermann K, Marschner K, Jensen A, Dermietzel R, Meier C (2010) The chemokine SDF-1/CXCL12 contributes to the 'homing' of umbilical cord blood cells to a hypoxic-ischemic lesion in the rat brain. J Neurosci Res 88:1223-1233.

Rossi SL, Keirstead HS (2009) Stem cells and spinal cord regeneration. Curr Opi Biotech 20:552-562.
Rossignol S, Schwab M, Schwartz M, Fehlings MG (2007) Spinal cord injury: Time to move? J Neurosci 27:11782-11792.

Rubin JB, Kung AL, Klein RS, Chan JA, Sun Y, Schmidt K, Kieran MW, Luster AD, Segal RA (2003) A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. Proc Natl Acad Sci USA 100:13513-13518.

Rudge JS, Manthorpe M, Varon S (1985) The output of neuronotrophic and neurite-promoting agents from rat brain astroglial cells: a microculture method for screening potential regulatory molecules. Brain Res 351:161-172.

Rudge JS, Alderson RF, Pasnikowski E, McClain J, Ip NY, Lindsay RM (1992) Expression of Ciliary Neurotrophic Factor and the Neurotrophins-Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Neurotrophin 3-in Cultured Rat Hippocampal Astrocytes. Eur J Neurosci 4:459-471.

Samdani AF, Paul C, Betz RR, Fischer I, Neuhuber B (2009) Transplantation of human marrow stromal cells and mono-nuclear bone marrow cells into the injured spinal cord: a comparative study. Spine 34:2605-2612.

Saporta S, Kim JJ, Willing AE, Fu ES, Davis CD, Sanberg PR (2003) Human umbilical cord blood stem cells infusion in spinal cord injury: engraftment and beneficial influence on behavior. J Hematother Stem Cell Res 12:271-278.

Sasaki M, Radtke C, Tan AM, Zhao P, Hamada H, Houkin K, Honmou O, Kocsis JD (2009) BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. J Neurosci 47:14932-14941.

Schiwy N, Brazda N, Müller HW (2009) Enhanced regenerative axon growth of multiple fibre populations in traumatic spinal cord injury following scar-suppressing treatment. Eur J Neurosci 8:1544-1553.

Schmalenbach C, Müller HW (1993) Astroglia-neuron interactions that promote long-term neuronal survival. J Chem Neuroanat 6:229-237.

Schucht P, Raineteau O, Schwab ME, Fouad K (2002) Anatomical correlates of locomotor recovery following dorsal and ventral lesions of the rat spinal cord. Exp Neurol 176:143-153.

Schwab ME (2004) Nogo and axon regeneration. Curr Opin Neurobiol 14:118–124.

Schwab JM, Bernard F, Moreau-Fauvarque C, Chèdotal A (2005) Injury reactive myelin/oligodendrocyte-derived axon growth inhibition in the adult mammalian central nervous system. Brain Res Rev 49:295-299.

Shyu WC, Lin SZ, Yen PS, Su CY, Chen DC, Wang HJ, Li H (2008) Stromal cell-derived factor-1 alpha promotes neuroprotection, angiogenesis, and mobilization/homing of bone marrow-derived cells in stroke rats. J Pharmacol Exp Ther 324:834-849.

Segarra J, Balenci L, Drenth T, Maina F, Lamballe F (2006) Combined signaling through ERK, PI3K/AKT, and RAC1/p38 is required for Met-triggered cortical neuron migration. J Biol Chem 281: 4771–4778.

Seijffers R, Allchorne AJ, Woolf CJ (2006) The transcription factor ATF-3 promotes neurite outgrowth. Mol Cell Neurosci 32:143-154.

Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS (2010) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells 1:152-163.

Sheth RN, Manzano G, Li X, Levi AD (2008) Transplantation of human bone marrow-derived stromal cells into the contused spinal cord of nude rats. J Neurosurg Spine 8:153-162.

Shiote M, Nagano I, Ilieva H, Murakami T, Narai H, Ohta Y, Nagata T, Shoji M, Abe K (2005) Reduction of a vascular endothelial growth factor receptor, fetal liver kinase-1, by antisense oligonucleotides induces motor neuron death in rat spinal cord exposed to hypoxia. Neuroscience 132:175-182.

Silver J, Miller JH (2004) Regeneration beyond the glial scar. Nat Rev Neurosci 5:146-156.

Silverman WF, Krum JM, Mani N, Rosenstein JM (1999) Vascular, glial and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures. Neuroscience 90:1529-1541.

Simonen M, Pedersen V, Weinmann O, Schnell L, Buss A, Ledermann B, Christ F, Sansig G, van der Putten H, Schwab ME (2003) Systemic deletion of the myelin-associated outgrowth inhibitor Nogo-A improves regenerative and plastic responses after spinal cord injury. Neuron 38:201–211.

Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, Ratajczak MZ, Janowska-Wieczorek A (2006) Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells 24:1254-1264.

Sondell M, Lundborg G, Kanje M (1999) Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 19:5731-5740.

Stichel CC, Müller HW (1998) Experimental strategies to promote axonal regeneration after traumatic central nervous system injury. Prog Neurobiol 56:119-148.

Stumm RK, Zhou C, Ara T, Lazarini F, Dubois-Dalcq M, Nagasawa T, Hollt V, Schulz S (2003) CXCR4 regulates interneuron migration in the developing neocortex. J Neurosci 23:5123-5130.

Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA (2003) VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest 111:1843-1851.

Taga T (1996) Gp130, a shared signal transducing receptor component for hematopoietic and neuropoietic cytokines. J Neurochem 67:1-10.

Taylor L, Jones L, Tuszynski MH, Blesch A (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. J Neurosci 38:9713-21.

Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, Hill CE, Sparling JS, Plemel JR, Plunet WT, Tsai EC, Baptiste D, Smithson LJ, Kawaja MD, Fehlings MG, Kwon BK (2010) A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury. J Neurotrauma [Epub ahead of print]

Thuret S, Moon LD, Gage FH (2006) Therapeutic interventions after spinal cord injury. Nat Rev Neurosci 8:628-643.

Tolosa L, Mir M, Asensio VJ, Olmos G, Lladó J (2008) Vascular endothelial growth factor protects spinal cord motoneurons against glutamate-induced excitotoxicity via phosphatidylinositol 3-kinase. J Neurochem 4:1080-1090.

Tovar-y-Romo LB, Tapia R (2010) VEGF protects spinal motor neurons against chronic excitotoxic degeneration in vivo by activation of PI3-K pathway and inhibition of p38MAPK. J Neurochem 115:1090-1101.

Trapp T, Kögler G, El-Khattouti A, Sorg RV, Besselmann M, Föcking M, Bührle CP, Trompeter I, Fischer JC, Wernet P (2008) Hepatocyte growth factor/c-MET axis-mediated tropism of cord blood-derived unrestricted somatic stem cells for neuronal injury. J Biol Chem 283:32244-32253.

Trompeter HI, Abbad H, Iwaniuk KM, Hafner, M, Renwick N, Tuschl T, Schira J, Müller HW, Wernet P (2010) MicroRNAs miR-17, miR-20a, and miR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC. PLoS ONE, in press.

Tsuboi Y, Kakimoto K, Akatsu H, Daikuhara Y, Yamada T (2002) Hepatocyte growth factor in cerebrospinal fluid in neurologic disease. Acta Neurol Scand 106:99-103.

Tsuzuki N, Miyazawa T, Matsumoto K, Nakamura T, Shima K (2001) Hepatocyte growth factor reduces the infarct volume after transient focal cerebral ischemia in rats. Neurol Res 23:417-424.

Tuszynski MH, Gabriel K, Gage FH, Suhr S, Meyer S, Rosetti A (1996) Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. Exp Neurol 137:157-173.

van den Berk LC, Jansen BJ, Siebers-Vermeulen KG, Netea MG, Latuhihin T, Bergevoet S, Raymakers RA, Kögler G, Figdor CC, Adema GJ, Torensma R (2009) Toll-like receptor triggering in cord blood mesenchymal stem cells. J Cell Mol Med 13:3415-3426.

van Heeckeren WJ, Fanning LR, Meyerson HJ, Fu P, Lazarus HM, Cooper BW, Tse WW, Kindwall-Keller TL, Jaroscak J, Finney MR, Fox RM, Solchaga L, Forster M, Creger RJ, Laughlin MJ (2007) Influence of human leucocyte antigen disparity and graft lymphocytes on allogeneic engraftment and survival after umbilical cord blood transplant in adults. Br J Haematol 139:464-474.

Vercelli A, Mereuta OM, Garbossa D, Muraca G, Mareschi K, Rustichelli D, Ferrero I, Mazzini L, Madon E, Fagioli F (2008) Human mesenchymal stem cell transplantation extends survival, improves motor performance and decreases neuroinflammation in mouse model of amyotrophic lateral sclerosis. Neurobiol Dis 3:395-405.

Vrinten DH, Hamers FP (2003) 'CatWalk' automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. Pain 102:203-209.

Wang F, Yasuhara T, Shingo T, Kameda M, Tajiri N, Yuan WJ, Kondo A, Kadota T, Baba T, Tayra JT, Kikuchi Y, Miyoshi Y, Date I (2010) Intravenous administration of mesenchymal stem cells exerts therapeutic effects on parkinsonian model of rats: focusing on neuroprotective effects of stromal cell-derived factor-1alpha. BMC Neurosci 11:52.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941-944.

Wang Y, Deng Y, Zhou GQ (2008) SDF-1alpha/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. Brain Res 1195:104-112.

Weidner N, Ner A, Salimi N, Tuszynski MH (2001) Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proc Natl Acad Sci USA 98:3513–3518.

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

Weimann JM, Johansson CB, Trejo A, Blau HM (2003) Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. Nat Cell Biol 11:959-966.

Widenfalk J, Lipson A, Jubran M, Hofstetter C, Ebendal T, Cao Y, Olson L (2003) Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. Neuroscience 120:951-960.

Winter M, Wang XN, Däubener W, Eyking A, Rae M, Dickinson AM, Wernet P, Kögler G, Sorg RV (2009) Suppression of cellular immunity by cord blood-derived unrestricted somatic stem cells is cytokine-dependent. J Cell Mol Med 13(8B):2465-2475.

Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL (2001) Physiological migration of hematopoietic stem and progenitor cells. Science 294:1933–1936.

Wu S, Suzuki Y, Ejiri Y, Noda T, Bai H, Kitada M, Kataoka K, Ohta M, Chou H, Ide C (2003) Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. J Neurosci Res 72:343-351.

Yamashita YM, Jones DL, Fuller MT (2003) Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. Science 301:1547–1550.

Yang L, Bryder D, Adolfsson J, Nygren J, Månsson R, Sigvardsson M, Jacobsen SE (2005) Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. Blood 105:2717-2723.

Yoshihara T, Ohta M, Itokazu Y, Matsumoto N, Dezawa M, Suzuki Y, Taguchi A, Watanabe Y, Adachi Y, Ikehara S, Sugimoto H, Ide C (2007) Neuroprotective effect of bone marrow-derived mononuclear cells promoting functional recovery from spinal cord injury. J Neurotrauma 6:1026-1036.

Yu F, Kuo CT and Jan YN (2006) Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. Neuron 51:13–20.

Yu J, Li M, Qu Z, Yan D, Li D, Ruan Q (2010) SDF-1/CXCR4-mediated migration of transplanted bone marrow stromal cells toward areas of heart myocardial infarction through activation of PI3K/Akt. J Cardiovasc Pharmacol 55:496-505.

Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 106:1755-1761.

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

Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M (2003) Lack of enhanced spinal regeneration in Nogo-deficient mice. Neuron 38:213–224.

Zhu G, Huang L, Song M, Yu Z, Wu X, Zhao X, Jin J, Zhao G, Chen J, Yu S (2008) Over-expression of hepatocyte growth factor in smooth muscle cells regulates endothelial progenitor cells differentiation, migration and proliferation. Int J Cardiol 138:70-80.

Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393:595-599.

7. Abbreviations

AB	antibody
ACM	astrocyte conditioned medium
ALS	amyotrophic lateral sclerosis
Akt/PKB	protein kinase B
ATF-3	activating transcription factor-3
BBB	Basso–Beattie–Bresnahan (BBB) locomotor score
BDA	biotinylated dextrane amine
BDNF	brain-derived neurotrophic factor
BOS HL	base of support of the hindlimbs
CB-MSC	mesenchymal stem cells from umbilical cord blood
ChABC	chondroitinase ABC
CNS	central nervous system
CSPGs	chondroitin sulfate proteoglycans
CST	corticospinal tract
CXCL	CXC chemokine ligand
CXCR	CXC motif, alpha chemokine receptor
ctrl	control
DAPI	4,6´-Diamidino-2–phenylindol
DLK-1	delta-like 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DRG	dorsal root ganglia
E	embryonic day
ECM	extracellular matrix
ERK	extracellular receptor kinase
ESC	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
g	gram
GABA	gamma-aminobutyric acid

GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GMP	good manufacture practice
GVHD	graft-versus-host-disease
h	hour
HGF	hepatocyte growth factor
HLA	human leucocyte antigen
hNuc	human nuclei
HSC	haematopoietic stem cells
hUCB	human umbilical cord blood
IgG	immunglobuline gamma
IGF-1	insulin-like growth factor-1
Jak	janus kinase
kD	kilo Dalton
KIT	stem cell factor receptor, tyrosine kinase
Lgr5	leucine-rich-repeat-containing G-protein-coupled receptor 5
lHL	left hindlimb
LIF	leukaemia inhibitory factor
IGF-1	Insulin-like growth factor-1
IL	interleukin
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
max	maximal
min	minute
ms	mouse
MSC	mesenchymal stem cell
N2	cell culture medium for primary cortical neurons
NEF-H	neurofilament, heavy polypeptide
NEF-L	neurofilament, light polypeptide
NEF-M	neurofilament, medium polypeptide
NeuN	neuronal nuclei

NF	neurofilament
NGF	nerve growth factor
NGS	normal goat serum
NSC	neural stem cells
NT-3	neurotrophin-3
OB	olfactory bulb
OMgp	oligodendrocyte myelin glycoprotein
OSP	oligodendrocyte specific protein
PB	phosphate buffer
PBS	phosphate buffered saline
PDL	Poly-D-Lysin
Pen	penicillin
PFA	paraformaldehyde
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PNS	peripheral nervous system
PREF-1	preadipocyte factor-1
Rac1	ras-related C3 botulinum toxin substrate 1
Raf	rapidly growing fibrosarcoma, Serin/Threonin protein kinase
Ras	rat sarcoma, small G-protein
rbt	rabbit
rel	relative
rHL	right hindlimb
RI	regularity index
RMS	rostral migratory stream
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute, Institue where RPMI medium was composed
RST	rubrospinal tract
RT	room temperature

SC	spinal cord
s.c.	subcutaneous
SCF	stem cell factor
SCI	spinal cord injury
SDF-1	stromal cell-derived factor-1
SEM	standard error of the mean
SGZ	subgranular zone
Shh	sonic hedgehog
STAT	signal transducers and activators of transcription
Strep	streptavidin
SVZ	subventricular zone
Th	thoracic
Trk	receptor tyrosine kinase
Tuj1	β-III-tubulin
USSC	unrestricted somatic stem cells
USSC-CM	USSC-conditioned medium
VEGF	vascular endothelial growth factor
w	week
wpt	weeks post transplantation

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und unter ausschließlicher Verwendung der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die aus veröffentlichten und nicht veröffentlichten Schriften entnommen sind, habe ich als solche kenntlich gemacht.

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