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Bone Marrow Stromal cells for repair of the spinal cord

R.D.S. Nandoe Tewarie
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Bone Marrow Stromal cells for repair of the spinal cord

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

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28-08-1950 † 23-04-2004
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Outline of the thesis.


R.D.S. Nandoe Tewarie
A. Hurtado
R.H.M.A. Bartels
J.A. Grotenhuis
M. Oudega
A. Introduction to spinal cord injury

Each year, many people worldwide suffer from spinal cord injury (SCI). These injuries cause death of neural cells, severance and demyelination of descending and ascending axons, and, consequently, loss of motor and sensory function. Endogenous repair efforts fail to repair the spinal cord and, as a result, the functional impairments are permanent. Most people who experience SCI are destined to spend the remainder of their life in a wheelchair. Potential treatments for SCI are being tested in the clinic but so far none of these have emerged as one that reverses the devastating functional consequences of SCI. Here we review SCI with an emphasis on the current status of clinical care and clinical trials. In an accompanying review we discuss the application of stem cells for spinal cord repair.

Epidemiology and etiology of SCI

Inconsistent data reporting makes it difficult to accurately estimate the worldwide incidence of SCI. The annual incidence in the United States is about 40 cases per million population or about 12,000 cases per year. Over 77% of SCI occurred among males. A number of studies profiling the epidemiology of SCI indicated that the population of SCI people has grown over 255,000 (in 2007) with estimates between 227,080 and 300,938 patients. In the United States and most Western European countries, the average age at injury has increased over the last 3 decades from 28.7 to 39.5 years. Most injuries occur between the ages of 16 and 30. The percentage of people older than 60 that suffered from SCI has increased from 4.7% in 1980 to 11.5% among injuries since 2000.

In the United States, the main causes of SCI are motor vehicle crashes (42%), falls (27.1%), violence (15.3%), unknown (8.1%), sports (7.4%). These numbers are similar in other countries although the percentage of violence may be smaller. Over 70% of injuries are contusive injuries.

Consequences of SCI

Pathophysiological and anatomical consequences

A force to the vertebral column causes damage to the ligaments and vertebrae (Fig. 1A, B). The torn ligaments cause instability of the vertebral column. Dislocated bone fragments of damaged vertebrae may compress the spinal cord (Fig. 1A, B). This causes immediate neural cell death, axon damage and demyelination (Fig. 2A). The cellular damage results in instant loss of motor and sensory function. After the first destructive events, a sequence of molecular and cellular pathophysiological events (Fig. 2A) including an aggressive inflammatory response within the damaged tissue leads to additional tissue loss at the injury epicenter and at distant sites (secondary injury). On the other hand, there are also various cellular
events during the early and later stages of SCI that could be interpreted as attempts to correct for the inflicted damage (Fig. 2B).

**Functional consequences**
The functional consequences of SCI are highly variable and depend on the degree of tissue damage, which in turn depends on the impact severity. In patients with SCI with a relatively small amount of tissue damage, some endogenous recovery of function can be observed, which is most likely resulting from plasticity of the spinal nervous tissue. In people with SCI with large tissue damage the neurological deficits are generally major and permanent. There are very few reports of people with a large injury that regain motor function to a degree that independence can be achieved. In these few cases the injury was generally inflicted to the lower (lumbar) level of the spinal cord.

![Image](https://example.com/image1.png)

**Fig. 1. Imaging of human spinal cord compressive injury.** (A) Sagittal view of the cervical spinal cord on magnetic resonance imaging demonstrating the dislocated C5 and C6 vertebra compressing the spinal cord (arrow). The formation of a haematoma ventral to the spinal column C1-C4 indicates possible ligament damage (asterisk). (B) Closer view of the damaged C5 and C6 cervical vertebrae on computed tomography scan, clearly showing bony damage of the vertebrae, with dislocating fractures in the spinal canal (arrow). (C) Conventional X-ray lateral view of the cervical spinal column after dorsal stabilization from C2 to Th2. Because of the damage to tendons and vertebrae stabilization surgery needs to be implemented in a large number of cases of SCI. (D) Conventional X-ray antero-posterior view of the same patient with stabilized cervical vertebrae as in panel C.
Fig. 2. Degenerative and regenerative events after spinal cord injury. A schematic representation of the degenerative (A) and regenerative (B) events that take place after spinal cord contusion injury. Rostral is to the left. In B, the regenerative events that take place relatively early after injury and over a limited time period are upregulation of regeneration-associated genes (RAGs), axon sprouting, angiogenesis, trophic factor upregulation, Schwann cell invasion. Other regenerative events such as debris removal, stem cell birth/proliferation, myelination, and plasticity may also occur at later time points and over a longer time period.

Social consequences
The critical-care medicine practice for people with SCI has considerably improved during the last decade and is nowadays more widely available. Accordingly, more than 95% of SCI patients survive their initial hospitalization. SCI decreases the lifespan by about 7% each year.40 A functionally complete and high level (cervical) injury impact the lifespan more dramatically than a functionally incomplete or low level (thoracic-lumbar) injury.40 Together, the relatively young age when SCI occurs, the improved medical care, and the lack of effective therapies are responsible for the continually increasing number of paralyzed people with SCI. This puts a high financial burden on the patient, his/her family, and society.3, 13, 34 The Centre for Disease Control in the United States estimated that about 10 billion dollars are spent yearly on SCI treatment excluding the management of pressure ulcers, a common side-effect of SCI, which adds another billion dollars per year.363.
CLINICAL ASSESSMENT OF FUNCTION AFTER SCI
The American Spinal Injury Association (ASIA) impairment scale is often used to assess the level and the completeness of SCI. This scale grades the preserved dermatome for sensory function and the strength of 20 “key” muscles in the upper and lower limbs. It provides clinicians with a standard for grading sensory and motor function impairment after SCI. Table 1 provides the 5 ASIA scores and their implications. Testing the intrinsic foot muscle, could complement the ASIA score as, in the majority of SCI patients, it provides an earlier and superior indicator of supraspinal influence over motoneurons projecting to lower extremity muscles. Another frequently used scale is the ASIA Lower Extremity Motor Score (LEMS), an ASIA subscore, which provides a prediction of the ability to walk. The LEMS scale is commonly used together with and supplements the ASIA scale. A person without neurological deficits scores 50 on the LEMS scale. A score of 30 or more is predictive for community ambulation 1 year after injury and a score of 20 or less predicts limited ambulation. Classification of SCI can also be achieved by measuring functional ability using the Functional Independence Measure (FIM); a 7-point scale that measures 18 items concerning mobility, locomotion, self-care, bowel and/or bladder function, communication, and social cognition. A score of 1 indicates total dependence on a caregiver and a score of 7 indicates complete independence. Other scales to assess functional ability are the Quadriplegic Index of Function (QIF), Modified Barthel Index (MBI), and Walking Index for SCI (WISCI), Capabilities of Upper Extremity Instrument (CUE), Spinal Cord Independence Measure (SCIM) and the Canadian Occupational Performance Measure (COPM).
<table>
<thead>
<tr>
<th>ASIA grade</th>
<th>Classification</th>
<th>Level of impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Complete</td>
<td>No motor or sensory function preserved in the S&lt;sub&gt;4&lt;/sub&gt; and S&lt;sub&gt;5&lt;/sub&gt; segments</td>
</tr>
<tr>
<td>B</td>
<td>Incomplete</td>
<td>Sensory but not motor function preserved below neurological level and including S&lt;sub&gt;4&lt;/sub&gt; and S&lt;sub&gt;5&lt;/sub&gt; segments</td>
</tr>
<tr>
<td>C</td>
<td>Incomplete</td>
<td>Motor function preserved below neurological level and more than half of key muscles below that level have a muscle grade of &lt; 3</td>
</tr>
<tr>
<td>D</td>
<td>Incomplete</td>
<td>Motor function preserved below neurological level and at least half of key muscles below that level have a muscle grade of ≥ 3</td>
</tr>
<tr>
<td>E</td>
<td>Normal</td>
<td>Motor and sensory functions are normal</td>
</tr>
</tbody>
</table>

Table 1. Standard neurological classification of spinal cord injury. The presence of motor and sensory function per dermatome (neurological level) can be tested with the ASIA (American Spinal Injury Association) scale. For motor function, 10 key muscles in all four limbs are scored 0-5 (0 = total paralysis; 5 = normal) for a maximum score of 100. For sensory function, light touch and pin prick are being used at key sensory points on the right and left side of 28 dermatomes to assess the absence (score will be 0), impaired presence (1) or normal presence (2) of sensory function. The maximum sensory score is 224 (112 for each of the tests; 56 for each side). The scoring sheet can be found at http://www.asia-spinalinjury.org/publications/2006_Classif_worksheet.pdf

TREATMENT OF SCI
An acute and a chronic phase can be distinguished after SCI. Since SCI is often a consequence of severe accidents, clinical care during the acute phase is generally focused on stabilization of the patient. During the chronic phase the main attention will need to be on preventing and, if unsuccessful, treating SCI consequences such as pain, infections, and pressure ulcers among others.

Clinical care acutely after SCI
To date there is insufficient evidence that would support standards of care during the acute phase of SCI. It is advised to maintain patients in an intensive care unit for close monitoring of respiratory and hemodynamic complications. For adequate spinal perfusion, which is at risk due to injury-induced edema, a mean arterial pressure of 85-90 mmHg should be maintained. Depending on the type of injury, surgical interventions should be considered to relieve the spinal cord from compressing bone fragments. The physician may decide to...
perform surgery to decompress or stabilize dislocated vertebrae and the vertebral column (Fig. 1C, D). Decompression surgeries\(^49, 126\) may accelerate functional improvements and result in shorter hospitalization and rehabilitation periods\(^264, 316\). However, it does not necessarily result in an improved final outcome\(^71\).

The lack of standards of care during the acute phase of SCI is in part due to the large variability among injuries and makes its early management complicated. If bone fragments continue to compress the spinal cord, early surgery may be vital to prevent exacerbation of spinal cord tissue destruction. However, in cases without a clear sign of such urgency there is no consensus on whether and what type of early surgical/clinical interventions must be implemented\(^126\). The lack of standards of care is demonstrated by a case presented in figure 1. Due to a fall this patient had multiple fractures of the cervical spinal cord, including dislocation fractures of the C5 and C6 vertebrae resulting in compression of the spinal cord. In the acute phase, the patient was admitted at the intensive care unit and monitored. At first, a decompression laminectomy and dorsal spondylodesis from C2-Th12 was performed. However, the C5 dislocation fracture was not repositioned sufficiently requiring a second surgery where a corporectomy C5 and C6 including a ventral spondylodesis was performed. The type of surgical intervention should be considered on a case-to-case basis, which makes it complicated to study the efficacy of intervention in the acute phase after SCI in randomized and controlled clinical trials.

Besides surgical interventions, pharmacological treatments to limit the secondary injury after SCI are often considered. The best-known treatment is a high dose of the glucocorticosteroid, methylprednisolone sodium succinate (MPSS) within 8 hours after the injury\(^44, 46\). Experimentally it was demonstrated that a high dose of MPSS reduces the inflammatory response and limit tissue loss after damage to the spinal cord\(^311\). The effects of MPSS in patients with SCI were investigated in 3 consecutive National Acute Spinal Cord Injury Studies\(^44, 46\) (NASCIS). The results demonstrated that MPSS treatment in the acute phase of SCI resulted in neurological improvements up to 6 months after injury. MPSS was the standard of care in the United States and other countries. After a thorough review of the results from the NASCIS studies and a more comprehensive assessment of the benefits and risks involved in high dose MPSS treatment, the therapeutic benefits became disputed\(^64, 225, 287, 388, 344, 405\). Especially in patients with complete SCI high dose steroid treatment can lead to adverse effects such as myopathy and wound infection that may negatively influence functional outcome and in some cases may be life-threatening\(^25, 344\). Currently, many SCI clinics worldwide have discontinued the 'standard' acute administration of MPSS after SCI. The debate on the use of MPSS should be accompanied by efforts to develop alternative treatments that counteract the early destructive events occurring during the acute phase of SCI.
Clinical care at later stages after SCI: Preventing complications

Different complications may occur during the later stages of SCI (Fig. 3) that each demands specific actions and/or interventions. For instance, SCI can lead to pain\textsuperscript{439, 440}, decreased fertility\textsuperscript{327}, and autonomic dysreflexia with loss of bladder and bowel control\textsuperscript{436}. It has to be taken into consideration that many SCI patients get accustomed to the specific injury-related pain they experience and as a result reveal their distress to their physician often at a late stage\textsuperscript{350, 374}. For some SCI-related conditions, such as decreased fertility, it is the patient's personal desire that should guide the physician's actions.

Other common problems that arise after SCI are septicemia, respiratory insufficiency, and pneumonia due to muscle atrophy (Fig. 3). These complications may cause clinical deterioration and could eventually result in death. They often occur without typical symptoms. For example, pyelonefritis can occur without flank pain or a femur fracture can occur without pain. This may lead to delay or errors in diagnosis and treatment\textsuperscript{194, 196}. It is imperative that SCI patients receive annual screenings and long-term follow-ups to prevent these secondary complications. It is advised to treat patients on a regular basis with pneumococcal and influenza vaccine to prevent opportunistic infections\textsuperscript{201, 202}. Monitoring the skin and urinary tract and implementing aggressive treatments against pressure ulcers and urinary tract infections is needed to reduce the risk of septicemia\textsuperscript{201, 202}. Appropriate nutrition and exercise should also be incorporated in the (new) lifestyle. Rehabilitation programs should be implemented to reduce the risk of cardiovascular disease\textsuperscript{693}.

Generally, the possible medical complications of SCI patients are known, mostly recognizable, and their treatment often straightforward. It is different for the psychological problems that arise after SCI\textsuperscript{37, 397}. It may be possible to recognize some of these but treatment and responses to the treatment are depending greatly on the individual. One can expect an initial period of denial and/or inability to fully comprehend the functional consequences caused by the injury. Next a period of acceptance will have to run its course\textsuperscript{37, 397}. The patient needs to learn to live with the disabilities and this may be accompanied by bouts of depression. The mental state of the patient can have its effect on medical treatments\textsuperscript{439}. The psychological consequences of SCI should not be underestimated and appropriate guidance of patient and family should have an important place in the late care management of SCI\textsuperscript{37, 441}. 
The most common complications that occur during the first years after SCI are listed on the left and those that occur mostly at later (chronic) stages are listed on the right. Spasticity and pressure sores occur during the first years but are also common at chronic stages of SCI (UTI = urinary tract infection).

**Fig. 3. Complications after spinal cord injury.** The most common complications that occur during the first years after SCI are listed on the left and those that occur mostly at later (chronic) stages are listed on the right. Spasticity and pressure sores occur during the first years but are also common at chronic stages of SCI (UTI = urinary tract infection).

**CLINICALLY TESTED APPROACHES TO ELICIT FUNCTIONAL RECOVERY**

Continuing medical care after SCI is necessary to maintain the patient’s health and quality of life. However, this generally does not result in dramatic improvements in function that would allow the patient to live an independent life. Repair-promoting pharmaceutical and/or surgical interventions will be necessary to significantly change the functional outcome after SCI (Fig. 4). Here we will review some of the current treatments that are aimed at limiting functional loss and/or improving outcome after SCI. In addition we discuss possible future treatments for spinal cord repair. Table 2 provides a list of clinical treatments for SCI.
Neuroprotective approaches

During the last 30 years, many experimental studies have targeted neuroprotection (i.e., tissue sparing) early after SCI to improve outcome. Experimental evidence has shown that the functional loss after SCI can be limited by implementing neuroprotective approaches. The best known neuroprotective approach is acute administration of MPSS. This has been tested clinically and is still being used around the world\(^{44-46}\). MPSS treatment after SCI was first thought to improve functional outcome, but at present its true therapeutic potential is intensely debated\(^{225, 287, 288, 344, 425}\). The main goal of MPSS treatment after SCI is to decrease the aggressive inflammatory response normally present within the damaged tissue. This would limit the contribution of macrophages and activated microglia to the secondary loss of nervous tissue.

Another example of a molecule that could elicit neuroprotective effects after SCI is the tetracycline derivative, minocycline\(^{18-20}\). Minocycline may exert its protective effects through mechanisms that decrease injury-induced glutamate-mediated excitotoxicity\(^{18, 421}\) and/or immunomodulatory mechanisms such as blocking microglial activation\(^{19, 100}\). Moreover, minocycline may reduce oligodendrocyte and neuronal apoptosis as well as dieback of damaged axons\(^{139, 359}\). These experimentally studies have established minocycline as a promising candidate for early treatment after SCI. Currently, a phase I/II clinical study is underway in the United States to assess the efficacy of intravenously administered minocycline in the acute phase after SCI\(^{21}\).
Introduction and Outline Thesis

Axon regeneration after spinal cord injury. Schematic representation of axon regeneration that could contribute to functional recovery after SCI. There are 3 types of damages that are inflicted to axons after SCI. Axons may be still in contact with their target neurons but demyelinated (A) due to immediate or delayed death of oligodendrocytes. These axons can become ‘functional’ and contribute to motor recovery when they are remyelinated (A’) by either endogenous oligodendrocytes derived from local stem cells or oligodendrocyte precursor cells, or by transplanted stem/precursor cells or Schwann cells. Axons may be severed and thereby devoid of contact with their target neurons and demyelinated (B). In that case, the axons need to regenerate across/beyond the injury, establish synaptic contacts with target neurons, and be myelinated by endogenous or transplanted cells (B’). Unmyelinated axons may be severed and without contact with target neurons (C). These need to regenerate and establish synaptic connections with the original or new target neurons (C’).

Fig. 5. Axon regeneration after spinal cord injury. Schematic representation of axon regeneration that could contribute to functional recovery after SCI. There are 3 types of damages that are inflicted to axons after SCI. Axons may be still in contact with their target neurons but demyelinated (A) due to immediate or delayed death of oligodendrocytes. These axons can become ‘functional’ and contribute to motor recovery when they are remyelinated (A’) by either endogenous oligodendrocytes derived from local stem cells or oligodendrocyte precursor cells, or by transplanted stem/precursor cells or Schwann cells. Axons may be severed and thereby devoid of contact with their target neurons and demyelinated (B). In that case, the axons need to regenerate across/beyond the injury, establish synaptic contacts with target neurons, and be myelinated by endogenous or transplanted cells (B’). Unmyelinated axons may be severed and without contact with target neurons (C). These need to regenerate and establish synaptic connections with the original or new target neurons (C’).

Axon growth-promoting approaches

Functional improvements after SCI could be elicited by axon growth-promoting approaches as this could result in either restoration of damaged axonal circuits or elicit plastic events36, 16, 36 (Fig. 5). Examples of axon growth- or plasticity-promoting treatments are the administration of Cathrin® or NOGO antibodies. Cathrin® is a Rho antagonist that reduces the levels of intracellular GTPase-associated signaling proteins Rho and Rac to physiological levels16, 443. Elevated Rho has axon growth-inhibitory effects through the above mentioned pathway294. NOGO antibodies neutralize axon growth-inhibitory effects of oligodendrocyte myelin-bound Nogo71, 77. Thus, both Cathrin® and NOGO antibodies may result in enhanced axon growth and/or axon plasticity after SCI. Both treatments are currently tested clinically for their efficacy to repair the injured spinal cord77.

After SCI axons may still be intact but not functional due to injury-induced conduction block. Administration of the potassium channel blocker, 4-aminopyridine (4-AP) may restore such a conduction block and this could restore axon function and thus contribute to improved
function. The efficacy of 4-AP in SCI patients has been tested clinically but so far the outcome has been modest\textsuperscript{95, 112, 157}.

**Cell transplantation-based approaches**
Neuroprotection as well as axonal regeneration could be achieved by transplanting growth-promoting cellular or a-cellular substrates. Examples of cellular substrates that are clinically tested are olfactory ensheathing cells, peripheral nerves, and activated macrophages. Grafting olfactory ensheathing cells into the spinal cord is being examined in China, Australia, and Portugal\textsuperscript{94, 127, 231}. Autologous peripheral nerves are being grafted into the injured spinal cord in Taiwan\textsuperscript{80, 173} and activated autologous macrophages in Israel\textsuperscript{378} and Belgium\textsuperscript{209}. Thus far, there is no clear evidence that these transplantation strategies elicit major functional changes.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Main objectives</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elicit neuroprotection</td>
<td>Limit cell/tissue loss</td>
<td>*MP, *minocycline, riluzole</td>
</tr>
<tr>
<td>Elicit axon regeneration</td>
<td>Promote growth, myelination,</td>
<td><em>Cetrin</em>, *NOGO</td>
</tr>
<tr>
<td>Provide growth substrate</td>
<td>Support axon regeneration</td>
<td>SCs, *OEG, *PNG</td>
</tr>
<tr>
<td>(transplantation)</td>
<td>Promote cell survival / axon growth</td>
<td>*Activated macrophages</td>
</tr>
<tr>
<td>Facilitate plasticity</td>
<td>Promote formation new circuits</td>
<td><em>Cetrin</em>, *anti-NOGO-A</td>
</tr>
<tr>
<td>Restore conduction block</td>
<td>Increase axon excitability</td>
<td>*4-AP</td>
</tr>
<tr>
<td>Limit spasticity</td>
<td>Decrease reflex activity</td>
<td>*Baclofen, Fampridine</td>
</tr>
<tr>
<td>Limit osteoporosis</td>
<td>Prevent fractures</td>
<td>Risedronate, vibration</td>
</tr>
<tr>
<td>Improve bowel/bladder</td>
<td>Limit uncontrolled release</td>
<td>Gut stimulants, Ditropan</td>
</tr>
<tr>
<td>Alleviate (neuropathic) pain</td>
<td>Decrease hyperexcitability</td>
<td>Colostomy tube</td>
</tr>
<tr>
<td>Manage infertility</td>
<td>Facilitate erection, ejaculation</td>
<td>Amitriptyline, botulinum toxin</td>
</tr>
<tr>
<td>Elicit neuroprotection</td>
<td>Limit cell death</td>
<td>Sildenafil, vibration, levitra</td>
</tr>
<tr>
<td>Add/silence repair genes</td>
<td>Promote regenerative events</td>
<td>Hypothermia</td>
</tr>
<tr>
<td>Cell replacement</td>
<td>Enhance effects other interventions</td>
<td>Gene therapy: Viral vectors</td>
</tr>
<tr>
<td>Elicit muscle strength/use</td>
<td>Replace the lost neural cells</td>
<td>siRNA</td>
</tr>
<tr>
<td>Enable movements</td>
<td>Increase proliferation endogenous cells</td>
<td>Stem cells/progenitors</td>
</tr>
<tr>
<td>Enable movements</td>
<td>Prevent muscle atrophy</td>
<td>*A1T-O2 (Neotrofin)</td>
</tr>
<tr>
<td>Enable movements</td>
<td>Promote regenerative events?</td>
<td>Targeted physical therapy:</td>
</tr>
<tr>
<td></td>
<td>Facilitate (controlled) movements</td>
<td>*Locomat, *treadmill training</td>
</tr>
<tr>
<td></td>
<td>Facilitate muscle action</td>
<td>Robotic prosthetics</td>
</tr>
<tr>
<td></td>
<td>Promote regenerative events?</td>
<td>Electrical stimulation:</td>
</tr>
</tbody>
</table>

**Table 2. Therapies for the injured spinal cord.** Most of the listed approaches are or have been extensively studied in experimental models of spinal cord injury. For each a few examples are given that are investigated in the laboratory or clinic (indicated by asterisks). The approaches listed under the line are comparatively novel.
Frontiers in treatment of SCI

A relatively new concept that does not focus on anatomical and/or functional repair but rather on supporting the patient to achieve some degree of independence is the use of robotics to enable execution of specific motor tasks. Currently, there are concerted efforts to employ cerebral (cortical) control for steering robotic devices in combination with micro-chip technologies that would enable fine-tuning of the robotic movements depending on the tasks.

Other comparatively novel approaches implement physical and/or electrical activity to elicit spinal cord repair. Although these approaches are generally designed to improve muscle strength/use, it has been hypothesized that these particular approaches could also elicit regenerative cellular events that could contribute to improved outcome. Moreover, locomotor activity and electrical stimulation may promote spinal cord repair via stimulation of plastic mechanism within existing axon circuits involved in motor function.

A relatively new concept within the more conventional field of cell-based approaches to repair the spinal cord is the transplantation of stem cells to either replace lost cells or elicit regenerative cellular events after SCI. Stem cells have been studied for their potential to restore degenerative diseases in the central nervous system, such as Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, and multiple sclerosis or in traumatic injuries such as transient brain trauma or SCI.

Stem cells hold promise for spinal cord repair, but their true potential has not yet clearly been shown. At this time, stem cell-based therapies are at an early stage, and the associated risks are still unclear. To enable future use of stem cells for therapeutic purposes, discussions on all related issues and especially the moral aspects need to be held today. As with any medical intervention, the questions to be asked are whether this approach is the most likely one to achieve success and whether the risks justify the benefits. The challenges are to further develop these concepts within current ethical and social boundaries to increase our knowledge and, through experimental research, evaluate if they provide any clinical benefit for patients.

B. Stem cell-based therapies

Stem cells proliferate, migrate, and differentiate to form organisms during embryogenesis. During adulthood, stem cells are present within tissues/organs including the central nervous system where they may differentiate into neurons. After the identification and characterization of stem cells, a great deal of interest has been given to their potential for treatment of spinal cord injury (SCI), traumatic brain injury, and degenerative brain diseases. Considering their characteristic abilities to self-renew and differentiate into any cell type in the body the therapeutic promise of stem cells is justified. Before effective therapies can be developed there are several issues that need to be addressed and resolved.
These issues range from increasing our basic knowledge about the stem cell’s biology to prevailing over moral concerns fueled by religious and/or political ideas.

**STEM CELL DEFINITIONS**

A stem cell is defined by its ability of self-renewal and its totipotency. Self-renewal is characterized by the ability to go through an asymmetric division in which one of the resulting cells remains a ‘stem cell’, without signs of aging, and the other (daughter) cell becomes restricted to one of the germ layers. A stem cell may become quiescent and at later stages reenter the cycle of cell division.

A true stem cell is a totipotent cell; it can become any cell type present in an organism. By many the zygote is considered to be the only true totipotent (stem) cell because it is able to differentiate into either a placenta cell or an embryonic cell. Others define the cells of the inner cell mass within the blastocyst as embryonic stem cells (ESC). These cells are pluripotent as they cannot become a placenta cell (Fig. 6). Besides ESCs, undifferentiated cells can be found among differentiated cells of a specific tissue after birth. These cells are known as adult stem cells, although a better term would be ‘somatic stem cell’ since they are also present in children and umbilical cords. There is ample evidence that adult stem cells are not restricted to a particular germ layer and can transdifferentiate. An important advantage of adult stem cells over ESC is that they can be harvested without destruction of an embryo. As a result, adult stem cells have gained ample interest for their application in a variety of disorders.

**DIFFERENTIATION**

The pluripotent stem cell differentiates into a multipotent cell of the three germ layers. These three layers are the ectodermal layer (from which skin and neural tissue originate), the mesodermal layer (connective tissue, muscle, bone and blood cells), and the endodermal layer (gastrointestinal tract and internal glandular organs) (Fig. 7). The multipotent cell differentiates into a unipotent cell of a particular cell lineage within its own germ layer. The unipotent cell is capable of becoming a cell type within that particular cell lineage. At the successive phases of differentiation (or determination), the resulting progeny are known as progenitor cells; ‘stem cell-like’ cells capable of self-renewal. Within the central nervous system, unipotent neural progenitors become the neurons and glial cells present in brain and spinal cord (Fig. 6).

In the classic embryology, the totipotent stem cell becomes unipotent through successive phases of fate restriction. The steps in this process were thought to be irreversible. However, recently it was shown in vitro that the fate of multipotent cells can be chanced to another
germ layer. This process is known as transdifferentiation. The unlimited potential of transdifferentiation prompted many investigators to obtain cells that normally derive from stem cells that are more difficult to harvest from stem cells that are easier to harvest. For instance, it is less complicated to harvest stem cells from skin or bone marrow than from the brain.

Transdifferentiation has often been shown using non-specific markers and ignoring possible artifacts due to culturing methods. Therefore, the existence of transdifferentiation is still debated. It should be kept in mind that forced differentiation into a cell from a lineage within an unnatural germ layer could result in abnormal phenotypes that after grafting could induce carcinogenesis.

Fig. 6. From embryonic stem cell to differentiated neural cell. Embryonic stem cells from the inner cell mass of the blastocyst are pluripotent and go through phases of differentiation that changes them into unipotent cells. Here this process is depicted for the generation of neural cells; oligodendrocytes, neurons, and astrocytes.
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Fig. 7. All tissues in an organism originate from the 3 germ layers. These layers are the ectoderm layer, endoderm layer, and the mesoderm layer. Neural cells that form the central and peripheral nervous system derive from the ectoderm.

**POTENTIAL OF STEM CELLS FOR SPINAL CORD REPAIR**

After SCI, endogenous regenerative events occur indicating that the spinal cord attempts to repair itself. Schwann cells, the myelinating and regeneration-promoting cell in the peripheral nervous system, migrate from spinal roots into the damaged tissue and myelinate spinal cord axons\(^{18, 40}\). The expression of regeneration-associated genes is increased in damaged neurons\(^{14}\). There is a surge in proliferation of local adult stem cells and progenitor cells\(^{198, 253, 278}\). However, axonal growth is thwarted by growth-inhibitors present on oligodendrocyte myelin debris and on cells that form scar tissue\(^{44, 389, 420}\). Also, the new-born stem cells and progenitor cells do not integrate functionally into the injured spinal cord tissue. Thus, the endogenous regenerative events that occur after injury fail to repair the spinal cord.

Improved functional outcome after SCI may be elicited by neuroprotective approaches that limit secondary tissue loss and thus the loss of function. Alternatively, functional recovery could be elicited by axon growth-promoting approaches that result in restoration of damaged and/or formation of new axon circuits that could become involved in function. There is little doubt that stem cells and neural progenitor cells could become invaluable components of repair strategies for the spinal cord. They can become neural cells that may support anatomical/functional recovery. Alternatively, they may secrete growth factors that could support neuroprotection and/or axon regeneration. The potential of stem cells or progenitor
cells to support spinal cord repair has been investigated extensively\textsuperscript{90, 159, 414}. Their shortcomings for repair are also understood\textsuperscript{75, 471}. Over the last decade, stem cells have often been studied without implementing explicit criteria that would define the used cells as such. Consequently, the therapeutic potential of true stem/progenitor cells is still unknown. Other matters related to the use of stem/progenitor cells for SCI also need to be resolved before effective therapies can be developed. How can the cells be best obtained? Do they need to be differentiated \textit{in vitro} before transplantation? How can survival of grafted stem/progenitor cells be improved and uncontrolled division and differentiation be prevented\textsuperscript{192}? How can functional integration of the transplanted cells be improved?

**Cell replacement in the injured spinal cord**

Considering the ability of stem cells to become any cell type, their potential use for cell replacement strategies is commonsense. With the appropriate combination of (growth) factors (induction cocktail), ESC can be used to obtain neurons and glial cells\textsuperscript{31, 329}. ES-derived neurons can survive and integrate following injection into the injured rat spinal cord\textsuperscript{104}. It was shown that transplanted mouse ESC myelinate axons in the myelin-deficient \textit{shiverer} rat spinal cord\textsuperscript{43}. Also, mouse ESC grafted into the injured (normal) rat spinal cord result in improved functional recovery\textsuperscript{260}. Importantly, ESC were found to survive well within the injured spinal cord suggesting that long-term treatments could be achieved using this approach\textsuperscript{84}.

Human ESC can be directed towards multipotent neural precursors\textsuperscript{61}, motor neurons\textsuperscript{224, 228}, and oligodendrocyte progenitor cells\textsuperscript{200}. The latter were found to differentiate into mature oligodendrocytes \textit{in vitro} and \textit{in vivo}\textsuperscript{297}. Moreover, these cells are able to myelinate axons after transplantation into the spinal cord of myelin-deficient \textit{shiverer} mice and adult rats\textsuperscript{200}.

Neural progenitor cells (i.e., multipotent cells from which the cells of the central nervous system arise) often aggregate into neurospheres. Cao and colleagues\textsuperscript{58} showed that neural progenitor cells transplanted into the injured rat spinal cord favored differentiation into astrocytes. These results indicated the need for differentiation protocols prior to grafting\textsuperscript{59}. Fetal neural precursor cells genetically modified to express noggin, an antagonist of bone morphogenetic protein, differentiate preferably into neurons and oligodendrocytes\textsuperscript{353}. Transplantation of these cells into the injured mouse spinal cord resulted in improved functional outcome\textsuperscript{83}. However, this result could not be shown by others using the same approach\textsuperscript{122}.

Human neural progenitor cells can be harvested from blastocyst-stage embryos and manipulated to generate functional neurons and glia\textsuperscript{299}. When human neural progenitor cells were grafted into the injured rat spinal cord some of them were found to differentiate into oligodendrocytes\textsuperscript{92, 93}. Moreover, this finding was accompanied by improved functional outcome\textsuperscript{69, 91} (Fig. 8).
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Mesenchymal stem cells from bone marrow may also have therapeutic promise for SCI\textsuperscript{285, 322}. Although still debated\textsuperscript{66}, these particular adult stem cells have been shown to differentiate into bone, fat, tendon and cartilage cells\textsuperscript{337}. It has been published that these cells can also transdifferentiate in vitro into liver\textsuperscript{333}, skeletal\textsuperscript{19, 179, 432} and cardiac muscle\textsuperscript{249, 310} cells, and into central nervous system cells\textsuperscript{14, 47, 212, 270, 271, 333, 370}. This makes mesenchymal bone marrow stromal stem cells interesting for strategies for repair of the injured spinal cord. Many medical fields are exploring mesenchymal stem cells for instance for repair of the heart after myocardial infarction\textsuperscript{119, 365}, osteogenesis imperfecta in orthopedics\textsuperscript{68, 172}, organogenesis in internal medicine\textsuperscript{255, 307}, intervertebral disc disease in neurosurgery\textsuperscript{164, 366-368}, and stroke/neurodegenerative diseases in neurology\textsuperscript{222, 404}.

**Neuroprotection**

A neuroprotective strategy implemented soon after SCI would be the first line of defense against injury-induced tissue loss, and could contribute to an improved neurological outcome. It has been demonstrated that neural progenitor cells can protect against excitotoxicity\textsuperscript{233, 241}. Also, neural progenitor cells secrete a variety of molecules that could protect neural cells from death mechanisms other than excitotoxicity\textsuperscript{233, 241}. Thus, transplantation of these cells into the injured spinal cord could in fact exert neuroprotective effects. Bone marrow stromal cells have also been shown to elicit neuroprotective effects as grafting into the injured adult rat spinal cord resulted in tissue sparing\textsuperscript{199, 304}. This may have resulted from the secretion of a number of growth factors\textsuperscript{243, 219, 247, 457}.

**Axon regeneration**

Promoting axon growth in the injured spinal cord could contribute to restoring function. The ability of neural progenitor cells to secrete a variety of neurotrophic factors indicates that they could promote growth of damaged axons\textsuperscript{233, 241}. Adult neural progenitor cells were found to provide a permissive guiding substrate for corticospinal axon regeneration after spinal cord injury\textsuperscript{334}. The stem cell-like olfactory ensheathing cells assist axon regeneration in the injured spinal cord in a different manner. These cells are capable of preventing axons from recognizing growth-inhibitory molecules thereby allowing them to elongate into otherwise inhibitory terrain\textsuperscript{346, 349}.
Fig. 8. Potential effects of stem cells on spinal cord repair. Although transplanted stem cells could elicit axon regeneration and/or neuroprotection through secretion of growth factors, the most logical contribution to repair could come from their ability to replace lost neural cells. This could result in remyelination of de-myelinated axons if they become oligodendrocytes, restoration of (new) circuits if they become neurons, and providing scaffolding and nutrition of the injured area if they become astrocytes. Generally, the latter is not preferred because astrocytes express a number of axon growth inhibitory molecules that could prevent axon regeneration and thus limit the overall restoration.

CLINICAL APPLICATION OF STEM CELLS FOR SCI

The translation of approaches developed in the laboratory involving stem cells into the clinic is in progress. The use of stem cells harvested from tissue from an adult has facilitated the use of stem cells in the clinic as it has practically dismissed the moral objections surrounding the use of stem cells derived from an embryo. Nevertheless, for reasons described below the use of ESC is often preferred over that of adult stem cells. The use of human ESC for spinal cord repair in the United States has been proposed by Geron, a California-based biotechnology company. The application of adult human stem cells for treatment of SCI is in progress in many countries around the world. For instance, autologous bone marrow-derived stem cells have been transplanted in the injured spinal cord of 25 patients in Guayaquil, Ecuador, a trial that is supported by a California-based biotechnology company, PrimeCell Therapeutics LLC. Encouraging results have been reported such as improved walking and sensory perception. It has been suggested that surmounting the ethical hurdles (see below) could benefit the clinical application of ESC.
EMBRYONIC VERSUS ADULT STEM CELLS
ESC can develop into more than 200 different cell types present in the human body\textsuperscript{379} and under the appropriate circumstances into an entire organism\textsuperscript{35}. Human ESC have been isolated from blastocyst-stage embryos\textsuperscript{4}--\textsuperscript{9}. They have also been created using somatic cell nuclear transfer\textsuperscript{14, 15} or parthenogenetic activation of eggs\textsuperscript{45, 431}. Isolated ESC do not undergo senescence and retain high telomerase activity and normal cell cycle signaling, which explains their rapid proliferation in culture\textsuperscript{38, 350}. These plastic characteristics make the ESC suitable for central nervous system repair strategies. However, transplantation of ESC can result in teratomas due to uncontrollable cell proliferation\textsuperscript{350, 353, 386}. Also, ESC in culture may undergo genomic and epigenetic changes that could lead to transformation although this can be prevented using proper culture techniques\textsuperscript{467}. Transplanted ESC are prone to be rejected after injection into adult tissue and long-term treatment with immunosuppressive drugs may be required to prevent this loss\textsuperscript{350}. These findings have to some extent tempered the enthusiasm for application of ESC in repair strategies for the central nervous system, despite the fact that ESC possess by far the greatest potential and could be applied in a broad selection of reparative cell therapies.

An alternative for ESC are stem cells obtained from tissue after birth. For instance, neural progenitor cells have been harvested from adult brain\textsuperscript{234, 426} and spinal cord\textsuperscript{258}. However, adult stem cells are less plastic than ESC and divide less frequently in culture\textsuperscript{108}. Also, their differentiation potential may decrease in time\textsuperscript{419}. This makes them a possible but somewhat limited alternative for ESC. On the other hand, they offer the advantage that they can be transplanted without genetic modifications or pre-treatments. Immune rejection would not be an issue with adult stem cells when the cells are isolated from the patient (autografting). Also, adult stem cells show a high degree of genomic stability during culture\textsuperscript{35, 419} and usually do not result in tumor formation\textsuperscript{35}. Finally, there is much less moral concern surrounding the use of adult stem cells because they can be harvested from the patient. These latter features support the use of adult stem cells over ESC for strategies aimed at repairing the central nervous system. This is certainly true if strategies can be developed that circumvent the potential drawbacks of using adult stem cells such as the lower plastic ability and lower rate of proliferation \textit{in vitro} compared to ESC.

ETHICAL AND SOCIAL CONCERNS
One of the issues that surround the use of ESC is the time point at which we consider an embryo a person\textsuperscript{146, 335, 360}. According to the Roman Catholic Church and other religious institutions an embryo “must be treated from conception as a living person”\textsuperscript{361}. This implies that a blastocyst cannot be used to harvest. Others consider an embryo to be a person only after the 20th week of gestation\textsuperscript{145, 337} implying that ESC can be harvested from blastocysts. Also,
in that case, ESC could be harvested from embryos that were generated but not selected for in vitro fertilization. These would otherwise be discarded.

Discussions on what constitutes ‘life’ and when does ‘life’ start are often intense as they are driven by moral concerns fueled by religious and political ideas. These issues need to be addressed with respect to all opponents. Rules regarding the harvest and use of stem cells can only be set after full agreement by all groups within a society.

Ethical issues that surround the use of adult stem cells mostly involve their possible misuse. For instance, oocytes can be derived from stem cells of male origin which allows the production of a child from one or two male biological parents. The potential biological problems and psychological effects on the child are unknown. It would also be possible that the offspring develops defects due to acquisition of pairs of (recessive) genes.

Therapeutic cloning and genetic manipulation are other issues that surround the use of stem cells. Cloning of cells, genetically matched for the host, could in theory be beneficial for organ transplantation as it may solve issues such as organ shortage and rejection. Genetic manipulation could convert ESC into gametes, which would allow germ line gene therapy (GLGT).

**INDUCED PLURIPOTENT STEM CELLS**

It is now possible to obtain pluripotent cells by reprogramming differentiated cells, such as fibroblasts, via the introduction of 4 transcription factors, OCT3/4, SOX2, KLF4, and MYC (induced pluripotent stem (iPS) cells). This new technology was first described by Takahashi and Yamanaka for mouse fibroblasts and has now been applied for other mouse cells and for human somatic cells. Of the 4 transcription factors, MYC and KLF4 can be substituted by others. The underlying mechanisms for this typically straightforward and robust reprogramming procedure are still unknown and intensely debated. At present it is still unclear in how closely iPS-cells resemble conventional ESC and whether application of iPS-cells would result in similar functional results as can be obtained with ESC. Comparative gene-expression profiles of human ESC and human iPS-cells is now ongoing. Several hurdles need to be overcome before iPS-cell technology can produce cells for clinical use such as the use of retroviral vectors to introduce the transcription factors and the need for selection markers to identify the reprogrammed cells, as well as the use of the oncogene MYC and the integration of retroviral vectors into the genome. These needs are required for proper reprogramming but they modify the cell genetically and modified cells face regulatory obstacles for therapeutic applications. Nevertheless, it is evident that the iPS-cell technology is promising and has opened exciting avenues for the clinical application of pluripotent cells without the ethical obstacles that come along with the use of ESC.
C. Outline of the thesis

In Chapter 1 a general introduction to the etiology, clinical grading, and treatment options for spinal cord injury are reviewed in Part A. Aspects of stem cell terminology, advantages and disadvantages of stem cell application are reviewed in Part B. In Chapter 2 the literature on the use of the Bone Marrow Stromal Cell (BMSC), a specific type of adult stem cells, for repair of the injured spinal cord is reviewed. Transdifferentiation, neural induction based on morphology and electrophysiological properties are some of the aspects that are discussed. In Chapter 3 we compared the gene profiles of BMSC early and late in culture and discuss their potential for spinal cord repair in light of our results. In Chapter 4 we present an in vivo study, in which we have investigated BMSC survival as well as their neuroprotective effects in the contused adult rat spinal cord. Chapter 5 describes a study on the effects of different immunosuppressive agents on BMSC survival within the contused adult rat spinal cord. In Chapter 6 we studied whether a combination therapy of BMSC transplantation and immunosuppression would improve the overall outcome in term of locomotor and sensory function after a contusive injury in the rat spinal cord. Finally, in Chapter 7 and 8 the findings are summarized and discussed in light of future scientific and clinical perspectives.
Review of literature on the use of bone marrow stromal cells.

Bone marrow stromal cells for repair of the spinal cord: towards clinical application.

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INTRODUCTION

Stem cells are defined by their capacity for self-renewal and differentiation into different cell types. In the early embryonic phase, stem cells are totipotent but after a few divisions they are determined to become specific for one of the three germ layers; the ectodermal layer, which will give rise to skin and neural tissue, the mesodermal layer, which will give rise to connective tissue, muscle, bone and blood cells, and the endodermal layer, which will give rise to gastrointestinal tract and internal glandular organ cells. In the classic embryology, this ‘determination’ of the stem cells is thought to be an irreversible process. Recently, it has become clear that the determined stem cell is in fact phenotypically plastic and is able to give rise to cells from different germ layers, a process known as transdifferentiation.

Because of their versatility, stem cells have gained ample attention over the last years for their potential in replacement/repair approaches. However, the term ‘stem cells’ has been used loosely without clear and appropriate criteria that define the used cell types. For example, CNS-derived neurospheres have been used extensively as a source for neural stem cells (NSCs), whereas it is now clear that they are in fact heterogeneous cell populations consisting mostly of neural progenitors and precursors, i.e., cells that are already directed towards the neural lineage. Recently, Parker and colleagues elegantly demonstrated an overlap of 18% of stemness genes between CNS-derived neurospheres and the C17.2 NSC clone, which fulfills the in vitro and in vivo operational definition of a stem cell.

Interestingly, this percentage of overlap increased two-fold when the C17.2 NSC clone was cultured as a neurosphere reflecting a shift from a “stem-like” to a “differentiated” gene expression pattern.

The stroma of bone marrow houses multipotent cells that can differentiate into lineages of blood cells, stromal and skeletal tissue. It has been reported that these stem cells can also transdifferentiate into liver cells, skeletal and cardiac muscle cells, and CNS cells, but this is still debated. Bone marrow is relatively easy to obtain, which circumvents the ethical concerns that surrounds the use of embryonic stem cells. Because of its availability and its reported aptitude to transdifferentiate, stem cells from bone marrow are thought to serve as an alternative source for other types of stem cells that are needed for specific therapeutic approaches.

As with stem cells in general, there is much confusion regarding the correct terminology and abilities of cells derived from bone marrow. These cells have been referred to as “bone marrow stromal cells (BMSCs)” or “stromal cells”, because they reside in the stroma of bone marrow, or as “bone marrow stem cells” or “bone marrow-derived stem cells”, because a percentage of the cells have stem cell abilities. The cells have also been referred to as “mesenchymal stem cells” or “bone marrow-derived mesenchymal stem cells”, because of their origin from the mesodermal germ layer. Due to this confusing terminology it is difficult to have a clear understanding of the true identity of the cells used in the various studies.
addition, their ability to differentiate or transdifferentiate is unclear due mainly to the large variety of induction protocols used by different groups. For example, one group reported that about 1% of human and mouse BMSCs can be induced into the neural lineage, whereas another group using a different induction protocol reported that 80% of human and rat BMSCs could become neural cells. Clearly, different protocols lead to highly variable results and this makes it difficult to fully understand the abilities of the BMSC and thus its potential for therapeutic approaches.

The majority of groups working with cells derived from the stroma of bone marrow does not attempt to further isolate subpopulations and thus study a heterogeneous cell population that includes true stem cells as well as precursor and progenitor cells. Therefore, we propose that “bone marrow stromal cells” is the proper terminology for this collection of cells. We oppose that they are referred to as stem cells unless proper attempts have been made to isolate a homogenous subpopulation of clonally related cells that express known ‘stemness’ genes such as Nanog, Oct-4, and Myc. Moreover, we concur with Parker and co-workers, that stem cells in general are best defined operationally. Thus, the term ‘stem cell’ can only be applied when the cells are multipotent, able to populate a developing area or repopulate an ablated or degenerated area with appropriate cell types, able to be serially transplanted, and able to self-renew. For a more in-depth discussion on this operational definition for stem cells we refer to a previous publication.

HARVEST AND CULTURING OF BMSC

Although some small variations exist, BMSCs are harvested according to largely similar protocols among the many groups studying these cells for their potential in a variety of therapeutic approaches. Bone marrow cells are removed from usually long bones such as the femurs and tibiae by flushing with cold phosphate-buffered saline with low percentage of fetal bovine serum. These cells are washed and cultured in Dulbecco’s Modified Eagle’s Medium or Iscove’s Modified Dulbecco’s Medium with 10-20% fetal bovine and/or horse serum. After 3-5 days in culture, non-adherent cells, mainly red blood cells that have a short lifespan of about 72 h in these culture conditions, are removed, and the remaining cells washed and further cultured in the same medium. Usually within two weeks after initiation, the cultures consist of spindle-shaped cells with some monocytes and macrophages present. The adherent cells are removed by trypsinization and then replated for further expansion or used experimentally. These particular cells, i.e., the plastic adherent cells, are considered to be ‘the BMSCs’. Generally these cells are not further phenotypically characterized. However, several groups did analyze the presence of a battery of surface antigens and with great consistency demonstrated the presence on human BMSCs of MHC class I, CD13, CD44, CD63, CD73, CD29, CD90, CD105, and CD166 and the absence of MHC class II, CD14, CD45, and CD34.
Several other surface antigens (i.e., SH2, SH3, CD71, CD120a, and CD124) have been described for rat BMSCs. With this in mind we reviewed the literature on the use of BMSCs (harvested as described above) for repair of the spinal cord. We will focus primarily on the application of BMSCs and not only the stem cell fraction thereof. Nevertheless, through transdifferentiation the stem cell portion and possibly the precursors and progenitors after de-differentiation can give rise to cells from the neural lineage; neurons, astrocytes, and oligodendrocytes. Especially for smaller focal traumatic and demyelinating lesions it could be beneficial to acquire neural cells from BMSCs in vitro prior to transplantation into the spinal cord or manipulate them in vivo such that they can replenish lost neural cells.

DIFFERENTIATION OF BMSC INTO NEURAL LINEAGE IN VITRO

To get a better understanding of the true nature of BMSC-derived astrocytes, oligodendrocytes and neurons it is imperative to define criteria for each of them. Is it acceptable to merely assume that cells that express markers specific for a particular neural cell will also have relevant functional properties or should it be a requirement to demonstrate this at least in vitro? The expression of certain molecules has been accepted as an indication of differentiation into a particular neural cell type. Astrocytes express glial fibrillary acidic protein (GFAP) and oligodendrocytes express rat insulin promoter (RIP) and myelin-basic protein (MBP). Neurons are identified by the presence of β-3 tubulin (immature neurons), neuronal marker N (NeuN), neuron-specific enolase (NSE), neurofilaments (NF), and microtubule associated protein-2 (MAP-2). However, the expression of cell-specific markers alone is not adequate and, except for astrocytes, morphological characteristics that are in apparent agreement with a specific cell type can be misleading. Indisputably, the best criteria for a BMSC-derived neural cell are its functional properties, which unfortunately is much easier to demonstrate in vitro than in vivo. Nevertheless, unless BMSC-derived cells positive for RIP- or MBP myelinate central axons in vitro their designation as oligodendrocytes should be taken with caution. Similarly, unless BMSC-derived cells positive for neuronal markers have appropriate electrophysiological properties their designation as neurons should be carefully considered. In line with this, we propose to use the additive -like for cells that express particular markers but have not been functionally characterized. We believe that this would better reflect the uncertainty of the true nature of the particular cell.

Several groups have reported that BMSCs can differentiate into cells that express neuronal markers or into cells that have a neuron-like morphology. Figure 1A demonstrates rat BMSCs isolated and cultured according to earlier described methods. When brain-derived neurotrophic factor (BDNF) is added to the culture, the presence of neuronal-like cells can be observed (Fig. 1B). To benefit most from the ability of BMSCs to give rise to neural...
cells, it is imperative to investigate and optimize the culture conditions that are necessary for this transdifferentiation. Padovan and co-workers demonstrated that human BMSCs proliferated best and expressed the highest percentage of β3-tubulin (about 27% of the total population) when cultured in the presence of 20% fetal bovine serum and 10 ng/ml basic fibroblast growth factor (bFGF or FGF-2). With fibronectin as a growth substrate this percentage was further increased to approx. 48% \(^3\). BDNF or neurotrophin-3 (NT-3) elicited the expression of β3-tubulin up to over 40% of the cells, which could not be further increased by combining them with FGF-2 \(^4\). With these culture conditions, the cells did not express NeuN. In the same medium but without serum, about 10% of the cells differentiated into GFAP-positive astrocytes \(^4\). Unfortunately, the authors did not further combine these different culture conditions to possibly enhance the induction of BMSCs to differentiate into neuronal-like cells.

Although the study of Padovan and colleagues may suggest that serum is necessary for neural induction, nestin-positive neural precursor cells were found in serum-free culture conditions \(^4\). These nestin-positive cells differentiated into GFAP-positive astrocytes (based on morphology; approx. 40% of the population) or NeuN-positive neuronal-like cells (approx. 19%) after 5 days in co-culture with cerebellar granule cells \(^4\). The groups mentioned above reported elegant and comprehensive studies. Unfortunately, a general consensus for culture conditions for neural induction of BMSCs has not yet been established.

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**Fig. 1.** Panel A depicts undifferentiated rat BMSCs 7 days in culture expressing green fluorescent protein (GFP). The cells were isolated and cultured according to a previously described protocol and infected with lentiviral vectors encoding GFP. Addition of brain-derived neurotrophic factor pushes the BMSCs into neural-like cells as can be seen in panel B.
A number of studies have shown that BMSCs can be induced to become neural-like cells *in vitro* by adding growth factors, β-mercaptoethanol and dimethylsulfoxide in combination with butylated hydroxyanisole. Using these various induction protocols, 2-76% of the cells became neural-like cells. These results may indicate that depending on the growth factor the same intracellular pathway resulting in neural induction gets differentiaily activated. However, more likely is that the different growth factors exert their activity through different intracellular pathways that result in different degrees of neural induction.

Padovan and colleagues investigated whether among BMSCs there is a subpopulation that can more easily differentiate into the neural lineage. They compared unsorted BMSCs and a population of BMSCs sorted on the presence of CD133 on their membrane and demonstrated that a higher percentage of the latter was able to express neural markers. This phenomenon was also demonstrated by comparing gene expression patterns of the different subpopulations of BMSCs cells. Although this particular population of CD133-positive cells, or cells derived thereof, were not further defined functionally, these results make it clear that the BMSC population when obtained as described above is a heterogeneous cell population.

Interestingly, and strongly emphasizing that more complete criteria are imperative to define BMSC-derived neuronal cells, Lu and coworkers demonstrated that the neuron-like cells derived from BMSCs by adding β-mercaptoethanol to the culture medium are actually dying cells. Time-lapse microscopy revealed that the cellular extensions protruding from the cells are merely a result of cellular shrinkage. Lu and colleagues took this investigation one level further and demonstrated that these morphological changes of the BMSCs were actually due to cellular toxicity. They showed that cells exposed to several stressors, such as detergents, chloride and extreme pH, exhibited the same morphological characteristics, i.e., neuronal-like cells, as the BMSCs cultured in the presence of β-mercaptoethanol. Clearly, neural cells obtained *in vitro* from BMSCs need to be functionally characterized. In case of BMSC-derived neurons, demonstrating appropriate electrophysiological behavior is crucial.

**ELECTROPHYSIOLOGICAL ACTIVITY OF NEURON-LIKE CELLS**

So far only a few groups published *in vitro* evidence that BMSC-derived neuron-like cells have electrophysiological activity appropriate for neurons. Kohyama and colleagues demonstrated that such cells exhibited a resting membrane potential (Vrest) of -20 mV and -50 mV at 14 and 28 days *in vitro*, respectively. This was the first study that demonstrated that BMSC-derived neuron-like cells acquire a Vrest resembling that of neurons, which is approximately -70 mV. Jiang and colleagues cultured BMSC-derived neuron-like cells long-term with different mitogens and cytokines, then co-cultured them with fetal mouse brain...
astrocytes and demonstrated that the neuronal-like cells had a $V_{rest}$ between -8.4 and -55.4 mV. These authors also demonstrated that prolonged co-culture with the fetal astrocytes resulted in a further decrease of the resting membrane potentials. Moreover, these cells were then able to fire action potentials. Regrettably, this study did not investigate the potential of these cells to fire trains of action potentials, a characteristic of fully matured neurons.

Electrophysiologically active cells derived from BMSCs were also described by Wislet-Gendebien and colleagues. They reported that after 4-6 days in culture some of the cells demonstrated sensitivity to the neurotransmitters, GABA, glycine, serotonin, and glutamate, possessed an outward $K^+$ current but no inward $Na^+$ current, and exhibited a $V_{rest}$ of about -37 mV. These characteristics correspond with those described for neurons in stage 1 of their maturation. Wislet-Gendebien and colleagues further showed that after 7-15 days in culture the cells were able to fire a single-spike action potential and had acquired $V_{rest}$ of -56 mV (characteristics that corresponded to neurons in stage 2 of their maturation). These findings are exciting and demonstrate that cells within the BMSC population can differentiate in maturation stage 2 neurons when cultured under the appropriate conditions. It is unfortunate that Wislet-Gendebien and co-workers could not demonstrate the presence of fully mature neurons (stage 3), which are able to fire trains of spikes and exhibit a normal $V_{rest}$ of -70 mV. The results from the studies mentioned above indicate that in vitro the BMSC-derived neuronal-like cells acquire a more negative $V_{rest}$ in time. Perhaps they could have succeeded in creating fully matured neurons if they had cultured their cells for longer than 15 days. The differentiation of BMSCs into fully mature neurons in vitro remains one of the more intriguing challenges in the field of stem cells and CNS repair.

DIFFERENTIATION OF BMSC INTO NEURAL LINEAGE IN VIVO

The first study that provided evidence that BMSCs can differentiate into neural-like cells in vivo was from Mezey and Chandross. Using a mice model, they transplanted male bone marrow cells into the peritoneal cavity of female recipients. The grafted bone marrow cell preparation did not contain neuron- or glia-like cells at the time of transplantation, although it should be noted that about 18% of the cells expressed the neural precursor cell marker, nestin when cultured for several weeks. Using in situ hybridization techniques, Y-chromosome containing neurons were located in the brain of the host, suggesting that the grafted BMSCs had crossed the blood-brain-barrier and formed neurons within the CNS.

Interestingly, Cogle and colleagues also demonstrated Y-chromosome containing neurons that were nicely integrated in the hippocampus of three female humans that had received transplants of male bone marrow cells up to 6 years earlier. It should be mentioned that a fusion between a grafted BMSC and a host cell could result in false-positive results. In several studies it has been reported that BMSCs can spontaneously fuse with other cells
Whereas this is a real possibility, Cogle and co-workers used fluorescence in situ hybridization techniques to reveal the presence of only one X chromosome, concluding that in their study the neurons could not have been the result of cell fusion. The Y-chromosome-containing, transgender cells accounted for approximately 1% of all neurons and 1-2% of all astrocytes and microglial cells in the hippocampus. These studies provide exciting evidence that BMSCs can migrate across the blood-brain-barrier and differentiate into neural cells in the mature CNS, which is promising for the use of BMSCs in CNS reparative approaches.

BMSC FOR SPINAL CORD REPAIR

Spinal cord injury results in cell death, axonal damage, progressive loss of tissue, and impaired motor and sensory functions. Some restoration of function has been reported resulting from endogenous self-repair processes or from applied interventions. At present, due to the lack of repair approaches that cause meaningful functional restoration, spinal cord injury results in a wheelchair bound life. In general it is thought that functional recovery can be achieved by addressing several key areas; prevention of injury-induced cell death (neuroprotection) close and away from the injury, promoting axonal regeneration by decreasing the inhibitory nature of the environment at the injury site or by increasing the intrinsic ability of injured neurons to grow their axon, and promoting myelination of regenerated axons and demyelinated intact axons. It appears that a combination of these approaches followed by intensive rehabilitation to develop and stabilize new axonal circuits will be necessary. Moreover, the interventions need to be applied simultaneously and/or successively thereby creating optimal conditions for morphological and functional repair.

A typical feature of the injured cord is the progressive loss of the central gray and peripheral white matter creating large fluid-filled cysts. To provide axons with a substrate to grow across these cavities transplantation of cells has been widely explored. Many cell types, alone or in combination, have been investigated over the last decades. Over the last years, the potential beneficial use of BMSCs in restorative approaches of the spinal cord has attracted ample attention. Table 1 provides an overview of studies in which BMSCs were applied into the damaged spinal cord and the results that were obtained. Clearly, among these studies some results are confusing and in disagreement with each other. For optimization of BMSC transplantation paradigms for application in clinical trials several crucial questions regarding cell survival, migration, neuroprotection, axonal regeneration and functional recovery need to be addressed.
Cell survival

An essential aspect for successful cell transplantation approaches is survival of the grafted cells. In vitro, BMSCs are cultured in medium containing 10-20% serum. Factors other than present in serum are not essential for their survival and proliferation. In fact, addition of growth factors such as BDNF, FGF-2, or neurotrophin-3 (NT-3) instigates differentiation of the BMSCs into neural-like cells rather than affect survival314. In vivo, in a rat contusion injury model, Hofstetter and colleagues170 showed that more BMSCs survived when transplanted one week after injury compared to immediately after injury. The surviving cells were located within trabeculae that span the injury site81, 170. However, with the one week delay only 1% of the cells (about 3000 total) survived at 4 weeks after grafting and although this is an increase over the percentage of cells that survived immediate transplantation (<0.15%) the total number of surviving cells was very low170.

It has been proposed that one of the mechanisms underlying death of cells transplanted into the spinal cord is injury-induced inflammation409, 455. The cellular and molecular components of the inflammatory response could initiate cell death28, which would also explain improved survival with delayed grafting paradigms. If this were the case, grafting into a chronically injured cord would further improve cell survival. Unfortunately, so far there have been no conclusive results on their survival rate in the chronically injured spinal cord. In one study, BMSCs grafted into a contusion site at 3 months after injury reportedly survived an additional 4 weeks but actual numbers were not provided472. From the data so far, it has become clear that the survival of the BMSCs is compromised after transplantation into the injured spinal cord.

Several recent publications have reported that in vitro BMSCs produce and secrete a variety of growth factors such as glial cell line-derived neurotrophic factor (GDNF)143, 457, nerve growth factor (NGF)143, 219, 247, BDNF74, 219, 247 and, albeit in smaller amounts, FGF-2247. These factors may have pronounced effects on repair-related processes such as neuroprotection and axonal outgrowth, but they may also affect BMSC survival and/or proliferation in vivo through an autocrine action457. If this is the case why then do BMSCs survive poorly within the injured spinal cord? It is possible that the grafted BMSCs simply do not secrete enough of the necessary growth factors to positively effect their own survival within an extremely harsh injury milieu with many cells and factors that negatively influence survival. Also, there may be batch-to-batch differences in the ability to produce growth factors, which was demonstrated for human BMSCs59 and that could result in highly variable results masking the true potential of BMSCs to survive the spinal injury milieu.

Clearly, for the development of safe and effective clinical application the survival of BMSCs after transplantation into an injury has to be improved. Especially when the cells also function to deliver factors that are most likely necessary to optimize the neuroprotective and axonal regeneration response. Future research should concentrate on decreasing death of
BMSCs within the lesion area, possibly by elevating local levels of growth factors essential for survival or by preventing the up regulation of apoptotic molecules or promoting the expression of anti-apoptotic molecules.

<table>
<thead>
<tr>
<th>Publication Source</th>
<th>Species</th>
<th>In vitro</th>
<th>Injury model</th>
<th>Number of cells</th>
<th>Main results</th>
<th>Additional results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hofstetter et al, 2002</td>
<td>rat</td>
<td>no</td>
<td>Contusion at T9 level. Cells grafted immediately or 7 dpi, 2 mm rostral and caudal. Survival 5 weeks.</td>
<td>300,000</td>
<td>Transplanted BMSCs form guiding strands at the injury site.</td>
<td>Better BMSC survival with delayed grafting (7 dpi). Motor outcome improved at 5 weeks.</td>
</tr>
<tr>
<td>Akiyama et al, 2002b</td>
<td>mice</td>
<td>no</td>
<td>EB-X lesion T10 level. Cells grafted 3 dpi. Rats were immunosuppressed. Survival 3 weeks.</td>
<td>5,000</td>
<td>Remyelination of demyelinated axons after BMSCs transplantation.</td>
<td></td>
</tr>
<tr>
<td>Corti et al, 2002</td>
<td>mice</td>
<td>no</td>
<td>Cells injected in tail vein after X-irradiation. Survival 3 months.</td>
<td>10,000,000</td>
<td>Systemically infused BMSCs migrate towards spinal cord.</td>
<td></td>
</tr>
<tr>
<td>Inoue et al, 2003</td>
<td>rat</td>
<td>no</td>
<td>EB-X lesion lumbar spinal cord. Cells grafted 3 dpi, systemic or focal. Survival 3 weeks.</td>
<td>100,000 - 10,000,000</td>
<td>Remyelination of demyelinated axons after BMSCs transplantation.</td>
<td>More myelinated axons with focal injection. Focal application more efficient.</td>
</tr>
<tr>
<td>Lee et al, 2003</td>
<td>mice</td>
<td>no</td>
<td>Contusion at T11 level. Cells grafted 7 dpi in and 2 mm rostrally to injury. Survival 4 weeks.</td>
<td>3,000</td>
<td>Neural differentiation: BMSCs differentiate into neurons in the brain and astrocytes in the cord.</td>
<td></td>
</tr>
<tr>
<td>Wu et al, 2003</td>
<td>rat</td>
<td>no</td>
<td>Contusion injury T9 level. Cells grafted immediately. Survival 1,3,4 weeks.</td>
<td>1,000,000</td>
<td>Neuroprotective: significant reduction of cavity volume.</td>
<td>BMSCs survival decreased in time up to 3 weeks. Motor outcome significantly improved up to 14 dpi.</td>
</tr>
<tr>
<td>Ankeny et al, 2004</td>
<td>rat</td>
<td>no</td>
<td>Contusion injury T9 level. 2 dpi transplantation of cells into lesion cavity. Survival 8 weeks.</td>
<td>60,000</td>
<td>Neuroprotective: BMSCs reduce cavity volume. Increased spared tissue volume and white matter.</td>
<td>No significant improvement in motor outcome after 8 weeks.</td>
</tr>
<tr>
<td>Lu et al, 2004</td>
<td>rat</td>
<td>no</td>
<td>BMSCs-Nt3 into lesioned dorsal column 5 days after cAMP in L4 DRG and NT3 immediate rostral to graft. Survival 3 months.</td>
<td>200,000</td>
<td>Axonal regeneration: cAMP and NT-3 combined promoted axonal growth of sensory axons.</td>
<td></td>
</tr>
<tr>
<td>Satake et al, 2004</td>
<td>Rat</td>
<td>No</td>
<td>Contusion 1-10 level. 3,7 dpi cell injection lumbar subarachnoid space.</td>
<td>1,000,000</td>
<td>Homing of transplanted cells towards lesion area.</td>
<td>Some BMSCs differentiated into nestin-positive immature neurons or glial cells.</td>
</tr>
<tr>
<td>Zurita et al, 2004</td>
<td>rat</td>
<td>no</td>
<td>Contusion at T7 level. Cells grafted 3 mo post injury at lesion site. Survival 4 weeks.</td>
<td>1,000,000</td>
<td>Significant improvement in motor outcome (BBB) after 2 weeks in the chronically injured rat.</td>
<td>BMSCs survive and form bridges in the cavity.</td>
</tr>
<tr>
<td>Sigurjonsson et al, 2005</td>
<td>human</td>
<td>no</td>
<td>In vivo surgery and cell implantation in chicken embryo (stage 15-16). Survival 4-9 days.</td>
<td>20,000</td>
<td>Transplanted cells indistinguishable from neurons chick and also electrophys. active.</td>
<td>During differentiation loss of CD14 expression by BMSCs.</td>
</tr>
<tr>
<td>Lu et al, 2005</td>
<td>rat</td>
<td>Yes</td>
<td>Dorsal column lesion C3. Survival up to 3 months.</td>
<td>100,000</td>
<td>Neural induced cells in vitro do not express neural markers in vivo.</td>
<td>BDNF secreting BMSCs led to higher axon density. No change in motor outcome at 3 months.</td>
</tr>
</tbody>
</table>

1 BMSCs were neurally induced following the procedure described by Woodbury et al., 2002 and modified to express BDNF.
2 EB-X lesion=Ethylbromide injection with X-irradiation to create a demyelinating injury.
Cell migration
Are BMSCs able to migrate towards or away from the site of injury/transplantation? In vitro studies have shown that BMSCs express CXCR4, the receptor for the chemokine, CXCL12 (also known as stromal derived factor-1, SDF-1). CXCL12 has been implicated in cell migration possibly through the extracellular signal-regulated kinase (ERK) and Akt phosphorylation pathways. Interestingly, under some pathological conditions reactive astrocytes produce CXCL12. It is possible that following spinal cord damage upregulation of the level of CXCL12 attract CXCR4-positive BMSC towards the injury site. This could be the mechanism at the basis of the homing of BMSCs into spinal cord injury sites. However, at this time it is not known whether this particular chemokine is present within the injured spinal cord.

In vivo, systemically administered BMSCs have been reported to migrate towards injury sites in the brain, but the results regarding homing towards the injured spinal cord have been conflicting. Recently, In-oxine-labeled BMSCs were shown to migrate poorly towards the injured spinal cord following intravenous administration. On the other hand, BMSCs labeled with iron-oxide microbeads were detected using magnetic resonance imaging within a spinal cord compression injury after intravenous administration. Satake and co-workers demonstrated that BMSCs grafted into the lumbar subarachnoid space aggregated onto the cord near a thoracic contusion injury site and that a few migrated into the contusion injury. Possibly, the meninges may have prevented more BMSCs to migrate into the spinal cord parenchyma. Obviously, if BMSCs are able to migrate towards an injury site in the adult spinal cord, it would allow for systemic delivery of the cells thereby avoiding invasive transplantation strategies.

Migration of transplanted BMSCs away from an injury site in the spinal cord might be beneficial for outgrowth of regenerating axons. It was reported that such migration did not occur in a contusion injury model. However, at present, the question whether BMSCs are truly capable of migration within the injured spinal cord has not explicitly been answered. Future research should focus on these questions because the outcome is crucial for the design of BMSC transplantation paradigms for clinical application to repair the injured spinal cord.

Neuroprotection
Although grafting of cells into the injured spinal cord is typically applied to generate a growth response, a neuroprotection effect can usually also be observed. Repeatedly, it has been demonstrated that cellular grafts limit the loss of nervous tissue in the injured cord. In fact, in animal models of spinal cord injury and repair improvements in motor performance seen after cell transplantation are often attributed to neuroprotection rather than axonal regeneration. Grafting BMSCs into the contused adult spinal cord also promotes tissue sparing, which was evidenced by smaller cavities and preserved host white matter.
Chapter 2

It is likely that the mechanism underlying the neuroprotective effect of BMSC transplants is related to the ability of the cells to produce and secrete factors that either arrest and/or prevent the onset of cell destructive events. BMSCs are known to produce and secrete factor (GDNF)\textsuperscript{143, 417}, nerve growth factor (NGF)\textsuperscript{143, 219, 247}, BDNF\textsuperscript{74, 219, 247} and, albeit in smaller amounts, FGF-2 \textsuperscript{247}. These factors have all been implicated in neuroprotective effects. NGF and BDNF increase survival\textsuperscript{4, 8} and decrease apoptotic death of neurons and oligodendrocytes\textsuperscript{39}. BDNF also increases oligodendrocyte proliferation\textsuperscript{267}. GDNF has been implicated in the rescue of motor neurons\textsuperscript{31, 78} possibly by activating MAP kinase and Bcl-2, an anti-apoptotic regulator\textsuperscript{28}. FGF-2 is known to positively effect tissue sparing\textsuperscript{187, 188, 269} and promote neuronal survival and angiogenesis\textsuperscript{269} following spinal cord injury. Another molecule produced by BMSCs that could positively influence tissue sparing is VEGF, a potent angiogenic factor\textsuperscript{158}.

**Axonal regeneration**

In a few studies the axonal regeneration promoting abilities of BMSCs have been addressed. Lu and co-workers\textsuperscript{244} demonstrated that transplantation of native BMSCs into the contused spinal cord promoted modest sensory and motor axon regeneration, whereas grafting of neurally-induced BMSCs did not result in axon growth. One explanation for the failure of the neurally-induced BMSCs to promote axonal regeneration in the injured spinal cord is that these cells die soon after transplantation. The neural induction of the BMSCs was performed according to an earlier described method\textsuperscript{32, 448, 449}, which, as had been already recognized by Lu and colleagues\textsuperscript{245}, causes BMSCs to die rather than become neuron-like cells.

An alternative explanation for the lower axonal growth response observed by Lu et al\textsuperscript{244} is that neurally induced BMSCs are less effective in eliciting such a response than undifferentiated BMSCs, for instance because they produce and secrete less growth-promoting factors. At present it is known that BMSCs produce and secrete several growth factors. However, it is unknown whether neurally induced BMSCs actually produce growth factors or whether they do so but in lower amounts than undifferentiated BMSCs. Indirect evidence that the neurally induced BMSCs do not produce enough growth factors to stimulate axonal regeneration was provided in two studies demonstrating that transplantation of neurally induced BMSCs genetically modified to produce and secrete BDNF did improve the axonal growth response\textsuperscript{244, 248}. In another study, a multifaceted and intriguing spinal cord injury/regeneration model was used to investigate the regeneration-promoting capacity of BMSCs\textsuperscript{244}. BMSCs modified to secrete NT-3 were transplanted in a transection injury of the mid-thoracic dorsal columns one week after administration of cAMP into the L4 dorsal root ganglion as a preconditioning stimulus for the sensory neurons. This was then combined with injection of NT-3 after injury/grafting within and beyond the injury site\textsuperscript{244}. The combination of all interventions resulted in regeneration of ascending sensory axons into and from the BMSC graft. Either cAMP or NT-3 administration alone did not result in such an axonal response.
These results suggested that a combinatorial approach that stimulates both the neural soma and axon might effectively increase the axonal regeneration. Surprisingly, in the study of Lu and colleagues244 application of cAMP alone at the level of the sensory neurons did not result in improved sensory growth while it had been implicated in such a response earlier292 as well as in other types of axonal regeneration responses293. Clearly, as with many other promising cell types for transplantation into the injured spinal cord, more extensive studies need to be performed before BMSCs can be used effectively in repair strategies.

**Functional recovery**

It has been reported that BMSC results in significant improvement of hindlimb locomotor performance when transplanted in the acutely451, sub-acutely43 and chronically472 contused spinal cord. In all three studies hindlimb function was evaluated using the open-field BBB-test, which scores for joint movements, paw placement, weight support, and fore/hindlimb coordination23. Although a valid way to test hindlimb function, the BBB test has limitations; the scoring is subjective and difficult for fore/hindlimb coordination. This affects the proper assessment of hindlimb motor performance. Other sensorimotor tests such as foot print, grid - and beam walking, and analysis of gait using the CatWalk® provide a more complete measurement of hindlimb function. In addition, it was unfortunate that these particular studies41, 451, 472 did not investigate whether the improvements in behavior were associated with an axonal regeneration response. Considering that in these studies the observed functional improvements appeared relatively soon after injury and transplantation, it seems that neuroprotective mechanisms3, 10, 304, possibly through secretion of growth factors, rather than axonal regeneration responses242, 244 were at the basis of the improvements.

So far studies on behavioral effects following intraspinal transplantation of BMSCs have used a variety of models in different species. In mice or rat different numbers of BMSCs were grafted acutely into the cervical244 or thoracic spinal cord244, 451, or sub-acutely43 or chronically472 in the thoracic spinal cord. Most models involved a contusion injury244, 451, others a partial transection model244. The survival period after transplantation as well as the studied end points varied among these studies. Given the major differences between these approaches, it is difficult to compare the respective results and thus to properly value the effects of BMSCs on functional recovery and axonal regeneration in the injured spinal cord so far.

This brings up the question whether there should be one particular model that should be used uniformly by groups that study the use of BMSCs in spinal cord repair. Is there a best model? We do not support the idea of only one model. In humans, the morphological and functional outcome following spinal cord injury is highly variable. Nervous tissue loss, axonal dieback, neuronal death, and scar formation depend largely on factors such as the site and degree of injury and the post-injury care. Applying one particular model will ultimately only
benefit a percentage of spinal cord injured patients. However, one particular in vivo model can be superior to another to answer a particular aspect of spinal cord injury and repair. For instance, neuroprotective effects of BMSC transplants can best be addressed in contusion injuries, whereas their effect on axonal regeneration is most reliably assessed in complete transection injuries.

The highly variable outcome after human spinal cord injury requires testing of BMSC transplantation (and of other approaches) in a variety of in vivo injury models. This would allow proper judgment whether following a certain injury BMSC grafting or an alternative intervention would be best. For understanding the potential of BMSCs for spinal cord repair it would be better to have certain studies independently repeated to confirm the results, which if that would be the case may establish BMSC grafting as the type of intervention best suited for a particular type of injury.

CLINICAL APPLICATION OF BMSC
There is considerable experience with the harvesting of BMSCs from the iliac crests of patients206. In the clinic, following chemotherapy and/or radiation therapy, the bone marrow microenvironment is damaged resulting in diminished or delayed hematopoiesis103, 266. Allogeneic marrow transplants have been explored for reconstitution of the damaged marrow stroma, although at present it seems that recipients of such transplants have only host-type marrow stromal cells after transplantation390, 407. Also, BMSCs are more increasingly used for surgical approaches for spinal fusion or for degenerated disc disease275, 434.

BMSCs have several features that make them appealing candidates for transplantation after spinal cord injury in the human which include the facts that (a) they can be relatively easily isolated under local anesthetic206, (b) human BMSCs can be rapidly and extensively expanded in cell culture13, 47, (c) there is no evidence that they produce tumors in vivo, even after immortalization to ensure an unlimited source of self-renewal ex vivo1, 115, (d) they have demonstrated capacity for tissue repair3, 244, and (e) they secrete growth factors in vivo that can enhance regeneration/repair73, 74. Clearly, BMSCs may be a good candidate for transplantation into the injured spinal cord. However, a concern is that a considerable variation exists among donors290, 291. Many factors that are difficult to control can influence the variations between donors, such as gender, genetic background, and general health. Therefore, we agree with Neuhuber and colleagues290, 291 that specific parameters need to be found that allow rapid and reliable selection of BMSCs with therapeutic potential.

Based on the above-mentioned features of BMSCs, there is much excitement about the potential use of these cells for spinal cord repair. However, it is also clear that BMSC are not the so-called “silver bullet”, the one therapy that will promote regeneration and restore function in the injured spinal cord. In fact, it is generally accepted in the field of spinal cord
injury and repair that such a “silver bullet” does not exist. Spinal cord injury is particularly complex and involves a variety of histopathological destructive and sometimes constructive events. A treatment for spinal cord injury aimed at repair of function needs to deal with all of these events in a timely, most likely sequential, fashion.

There have already been several clinical trials that have used intravenous administration of BMSCs for specific diseases such as patients with malignancies, but also efficacy trials in osteogenesis imperfecta, Hurler’s syndrome and metachromatic leukodystrophy. Each of these trials has had various degrees of success depending on the measured end-points. A positive outcome from all of these trials is that only one of the 68 patients that entered in the studies mentioned has suffered any side effects (i.e., mild urticaria as a reaction to the fetal bovine serum albumin in which the cells were grown), indicating that BMSCs can be used safely in clinical settings.

There is considerable debate as to which patients with a spinal cord injury would be the ideal candidates for testing cell types (including BMSCs) that have shown potential for clinical applications. Human trials approved by the United States Food and Drug Administration (FDA) require safety data prior to efficacy data. Consequently, most cellular transplantation strategies tested in clinical trials in the United States have focused on patients with functionally complete spinal cord injuries (ASIA A; American Spinal Cord Injury Association). However, for proper evaluation of the efficacy of transplantation strategies regarding functional improvements patients with incomplete lesions (represented by ASIA B-D) may be more beneficiary. Clinical trials transplanting BMSCs after spinal cord injury are ongoing in several countries including Korea, Mexico, Columbia and Brazil. Other than a Korean study results from these trials have not been published yet. In this particular study, six functionally complete spinal cord injured (ASIA A) patients received a BMSC transplant combined with the administration of granulocyte macrophage-colony stimulating factor (GM-CSF). All patients were operated on in the first two weeks after injury and a total of 1.8 ml with a density of $1.1 \times 10^6$ BMSCs/L was injected at the epicenter of a contusion lesion at 5 mm below the dorsal surface. In 5 out of 6 patients motor and sensory function improved, with 4 patients of them switching from the ASIA A to the ASIA C level. Follow-up evaluations up to 18 months after transplantation revealed no serious complications. Although these results are very promising, detailed information concerning neurological examination is lacking. Further studies in this arena will need to focus on reproducibility, safety and finally efficacy.

CONCLUDING REMARKS

Studies on the ability of BMSCs to transdifferentiate in vitro into the neural lineage often use different protocols. This leads to a great deal of confusion on the true capacity of these cells and thus their therapeutic potential. There clearly is a need for a consensus on how to
induce BMSCs into the neural lineage in vitro. Moreover, this procedure and in particular alternative ones that claim a higher efficiency need to be repeated independently. Also, there is a need for standardized criteria (operational definition)\textsuperscript{32} that will result in the proper designation of BMSC-derived cells as neurons or oligodendrocytes. Only then it will be possible to optimally benefit from the therapeutic potential of BMSCs for spinal cord repair.

\textit{In vivo}, the use of BMSCs in spinal cord injury models is still relatively new (see Table 1) and many questions remain unanswered. Some of these questions are: To what extent do BMSCs survive when grafted into the contused spinal cord and does time of transplantation make a difference? Do BMSCs migrate towards and away from the transplantation site and if so where to? To what extent do BMSCs differentiate when transplanted into the injured cord? Can grafted BMSCs support spinal cord repair and if so what mechanisms underlie the biological effects? Based on the reported differences between \textit{in vitro} and \textit{in vivo} results, it is clear that the influence of the milieu of the injured spinal cord on BMSCs is not fully understood. This is a complicated issue because of the abundance and variety of factors in injured nervous tissue all with different effects on BMSC survival, migration, and differentiation.

For transplantation purposes, it appears that differentiation of BMSCs prior to grafting would be best for effective repair for smaller, focal lesions of the spinal cord or for specific aspects in larger, more routinely seen, injuries. For instance, BMSC-derived oligodendrocytes could be used to address specifically a demyelinating disease such as multiple sclerosis or the effects of remyelination on functional restoration of the spinal cord after injury. Differentiation into neurons would allow addressing a motor neuron disease such as amyotrophic lateral sclerosis. However, is \textit{ex vivo} differentiation of BMSCs prior to grafting in the spinal cord necessary. In theory, the CNS environment may induce the cell to develop into the required cell type. Implantation paradigms using undifferentiated BMSCs \textit{in vivo} have shown promise in the laboratory and clinical trials are ongoing.
Gene expression pattern and suitability of bone marrow stromal cells.

Early passage Bone Marrow Stromal Cells express genes involved in nervous system development supporting their relevance for neural repair.

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Chapter 3

INTRODUCTION
The application of mesodermal bone marrow stromal cells (BMSC) for nervous system repair has been explored extensively. BMSC may promote repair through their ability to differentiate into neural cells and/or the secretion of growth-promoting molecules. The true potential of BMSC to repair nervous tissue is still under investigation. A more comprehensive understanding of the neural repair potential of BMSC and possibly of the underlying molecular mechanisms may be obtained by gene expression profiling. BMSC express genes common to cells from the mesodermal lineage such as bone, cartilage, and adipose cells. On the other hand, they also express genes that are typical for epithelial, endothelial, and neural cells, which may point at their proposed ability to differentiate across lineages.

Few groups have studied genes involved in nervous system development expressed by BMSC. It was shown that genes including neural progenitor genes are co-expressed in human-derived hippocampal neural stem cells and undifferentiated BMSC. Genes encoding for the neurotrophins nerve growth factor and brain-derived neurotrophic factor are expressed in undifferentiated BMSC and in neurally-induced BMSC, with reduced expression in the latter.

The expression of genes encoding for neurotrophins and proteins characteristic of neural progenitors in cultured BMSC may indicate their ability to support repair after transplantation into nervous tissue. To further evaluate the overall repair potential we profiled gene expression of BMSC from passage (P) 3, which are typically used for transplantation studies in our laboratory, using 44k whole genome rat microarrays. We also compared the gene profiles of P3 and P14 BMSC to investigate effects of long-term culturing on gene expression.

MATERIAL AND METHODS
BMSC isolation and culture
BMSC were isolated from Sprague-Dawley rats (8-10 weeks, 200-250g; Harlan, Indianapolis, IN, USA) as described previously. Briefly, rats were deeply anaesthetized and decapitated and their femurs and tibias immediately removed. The marrow from these bones was aspirated with a syringe, plated on plastic culture dishes, and cultured for 48-72 h in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen) at 37°C / 5% CO₂. Next, non-adherent cells were removed through washing with Hank's buffered salt solution (Invitrogen) and fresh culture medium was added to the adherent cells. These were grown to confluency and then harvested using 0.25% trypsin, diluted 1:2 in medium, plated, and cultured at 37°C/5% CO₂. BMSC were obtained from 3 rats and cultured in 4 different batches to ensure the availability of biological replicates for microarray analysis.
**RNA isolation and amplification**

From P3 and P14 BMSC, total RNA was isolated using Trizol-based and RNeasy Mini Kit RNA isolation methods. Cells were first homogenized in ice-cold Trizol (Life Technologies, Grand Island, New York). After phase separation by addition of chloroform, the aqueous, RNA-containing, phase was transferred to a new RNase-free 1.5ml tube and mixed with an equal volume of 70% RNase-free ethanol. Next, an RNeasy Mini column (Qiagen, Valencia, California) was used to isolate RNA according to the manufacturers’ guidelines. RNA yields and purity were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). RNA integrity was determined by the RNA Integrity Number (RIN) as measured by the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, California). RNA was amplified and a total of 500 ng cRNA labelled with Cy3 or Cy5 using the Agilent Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies) according to the manufacturers’ guidelines.

**Microarray design and hybridization**

For the microarray experiments we used 44K whole genome rat microarrays (Agilent Technologies) and a design consisting of direct comparisons between P3 and P14 BMSC (3 biological replicates each). Fragmentation and hybridization was performed according to the manufacturer’s guidelines (Agilent Technologies) using Agilent solutions and buffers. Briefly, target solution was prepared containing Cy3 and Cy5 labelled cRNA samples (500 ng in total). The cRNA was hydrolyzed for 30 minutes at 60 °C, followed by hybridization in hybridization buffer on the microarray probes for 17 h in a rotating chamber at 60°C. After hybridization, the slides were washed in wash solution 1 (6x SSPE, 0.005% N-Lauroylsarcosine) for 5 min and then in solution 2 (0.06x SSPE, 0.005% N-Lauroylsarcosine) for 1 min. The microarrays were then transferred to acetonitrile solution for 1 min and dried under pressurized nitrogen gas.

**Data analysis**

Microarrays were scanned with an Agilent microarray scanner. Red (Cy3) and blue (Cy5) intensity was acquired using Feature Extraction software v7.5.1 (Agilent Technologies). Arrays were normalized based on quantile normalization. Differential gene expression was determined by fitting a linear model to the normalized data. Nine microarrays were used for comparisons between P3 and P14 BMSC. Expression of a particular gene was accepted with an intensity of A>6.6, to exclude outliers based on background staining. Oversaturated genes were also removed from our analyzed set of genes. In the subset of genes, we looked for the presence of a clear neural relation in the name of the gene and for this we searched for neur*, glia*, oligo*, nerv* amongst others. Furthermore, we searched online in the present gene database for a known function in any neural processes.
Gene Ontology overrepresentation

Overrepresented Gene Ontology (GO)-classes were indentified using the web-based application GOstat (http://gostat.wehi.edu.au)\(^26\). We first determined the number of genes that were expressed in P3 as well as in P14 BMSC (cut-off: >2 x background intensity) and these served as a reference for the fraction of differentially expressed genes. For each top-level GO-term (e.g., biological process, cellular component, molecular function), we determined the overrepresented GO-classes. The overrepresentation of a GO-class within a set of differentially expressed genes means that this GO-class is statistically higher represented than would be expected by chance\(^26\). Statistical inference was calculated with Fisher's Exact Test. The Benjamini and Hochberg False Discovery Rate model was used to correct for multiple testing. The minimal length of considered GO-paths was 3. Significance was set at \(P < 0.01\).

RESULTS

BMSC have emerged as candidate cells for repair of the brain\(^{167, 273}\), spinal cord\(^{61, 354}\), and peripheral nerves\(^{17, 356}\). Profiling gene expression of BMSC may present information on their repair potential. Here we determined the gene expression profile of P3 BMSC, which we typically employ in our transplantation studies. Importantly, we have focused on genes associated with neural cells/events (searches for neur\(^*\), glia\(^*\), oligo\(^*\), nerve\(^*\) in their name) and on genes with a neural function as indicated in the gene database. In this study we have also compared gene expression of P3 and P14 BMSC to assess the effects of long-term culturing.

Gene expression profile in P3 BMSC

100 highest expressed genes

Among the 100 highest expressed genes, 55 genes encode for ribosomal proteins, 20 for proteins involved in protein binding processes, 6 for proteins involved in cell differentiation and proliferation, 4 for proteins involved in apoptosis, and 3 for proteins involved in enzyme activity (Fig. 1). The Notch3, Tubb2, and Gja1 genes were among the top 100 highest expressed genes. These genes are implicated in central nervous system development. Notch3 (Notch homolog 3) is involved in Notch signalling in cell differentiation\(^{11, 183, 256}\), forebrain development\(^{161, 256}\), and neuronal fate commitment\(^{193, 256}\). Tubb2 is a member of the tubulin superfamily which is implicated in nervous system development\(^8, 9, 236\). Gja1 (gap junction protein 1) is involved in protein and receptor binding in several processes including neuron migration and neurite morphogenesis\(^{170, 162}\).
Gene expression pattern and suitability of bone marrow stromal cells

Fig. 1. Schematic representation of the top 100 genes with the highest level of expression in bone marrow stromal cells at passage 3. The numbers in the graph indicate how many genes out of the top 100 encode for proteins known to be involved in the indicated respective processes.

Nervous system development
The genes expressed in P3 BMSC were studied for their involvement in nervous system development in general (Table 1A), oligodendroglial development and myelination (Table 1B), astroglial differentiation (Table 1C), and neuronal processes (Table 1D). These genes may be indicative of the potential of BMSC to become neural cells, and may thus be important for their potential to contribute to neural repair. Among those involved in nervous tissue development (Table 1A) were the genes that encode for nestin (Nes)147, 226, insulin-like growth factor 1 (Igf1)40, 79, and neural proliferation, differentiation, and control (Npdc1)117, 396. Genes implicated in oligodendroglial development and myelination (Table 1B) were Cog1, Erbb2, Mobp and Plp2 21, 171, 328, 375. P3 BMSC expressed genes involved in gliagenesis (Table 1C) such as Igf1, Pdgfra, and Erbb2 77, 230, 328 and in glia migration such as Cspg4 481, 462. Among those involved in neuronal processes (Table 1D) were genes implicated in cell proliferation and neurite development (glial cell line-derived neurotrophic factor, Gdnf)124 and synaptic plasticity (Ras homolog enriched in brain, Rheb)436.

Cell cycle and developmental processes
There were several genes involved in cell cycle and developmental processes expressed in P3 BMSC (Table 2; not including nervous system development genes as they are listed in Table 1). Among these were genes for stem cell factor KL-2 receptor binding (Kitl), which is important for proliferation of myeloid and lymphoid hematopoietic progenitors4, 352, insulin-like growth factor 1 (Igf1)40, 79, transforming growth factor beta (Tgfb3)43, 368, vascular endothelial growth factor C (Vegfc)48, 289, and growth arrest and DNA-damage-inducible 45 gamma (Gadd45g)485. We also identified the gene for adipose differentiation-related protein (ADRP) which is indicative for the mesodermal origin of BMSC202, 376.
Chapter 3

Growth factors and growth factor signalling
P3 BMSC expressed several genes encoding for growth factors or for proteins involved in growth factor signalling (Table 3). These may play a role in cell growth, differentiation and maturation and are thus potentially important for repair. Among those encoding for growth factors were the genes for vascular endothelial growth factor C (Vegfc)48, 489, glial cell line-derived neurotrophic factor (Gdnf)248, 381, and platelet-derived growth factor alpha (Pdgfa). Genes involved in growth factor signalling were the gene for fibroblast growth factor receptor-like 1 (Fgfrl1)398 and for neurotrophin receptor associated death domain (Nradd).

Cell death
We found several genes in the P3 BMSC that are involved in cell death (Table 4). These are of interest as they may be targets for improving BMSC survival after transplantation in the injured nervous system. Some of these genes are for members of the tumor necrosis factor superfamily, which is involved in cell death113, 411, and others are implicated in the caspase pathway (Casp2, Casp3, Casp6, Casp11), which is involved in apoptotic mechanisms50, 203.

Comparison between Gene expression profiles of P3 and P14 BMSC
We found that 6687 genes are expressed in P3 as well as in P14 BMSC. Of these genes, 159 were more than 1.5-fold higher expressed in P3 BMSC and 43 were more than 1.5-fold higher expressed in P14 BMSC. Thus, of the genes expressed in both P3 and P14 BMSC, 202 genes (3%) were differentially expressed and these genes were considered biologically relevant.

We found that from the 43 genes that were higher expressed in P14 than in P3 BMSC, 6 genes were over 3 times higher expressed (Table 5A). Among these were genes involved in cell death regulation (clusterin; Clu, and growth arrest-specific protein 2; Gas2)227, 381, and cell differentiation and proliferation (clusterin; Clu, and odd-skipped related 2 protein; Osr2)196, 384, 423.

From the 159 genes that were higher expressed in P3 than P14 BMSC, 12 genes were over 3 times higher expressed (Table 5B). Among these were genes involved in cell death (gremlin 1 homolog, Grem1)296, 474, brain development, motor axon guidance, and neuron migration (stromal cell-derived factor-1 gamma, SDF-1)282, 229, 303, and in cell survival (chemokine (C-C motif) ligand 2, Ccl2)283.

We used GOstat (http://gostat.wehi.edu.au) to perform functional data mining by GO-analysis on the 202 differentially expressed genes to identify overrepresented GO-classes (Table 6). The group of 6687 genes expressed in both P3 and P14 BMSC was used as a reference. From the 159 genes that were higher expressed in P3 than P14 BMSC, 85 (53%) were annotated in the GO database. We found a total of 43 GO-classes that were overrepresented including 29 in cellular metabolic processes and 9 in developmental processes and cell proliferation (GO:0048513/ GO:0007275/ GO:0048731/ GO:0032502/ GO:0048856/ GO:0035295/ GO:0001568/ GO:0001944/ GO:0008283). From the 43 genes that were lower expressed in
P3 than P14 BMSC, 22 genes (51%) were annotated in the GO database. Two GO-classes were overrepresented and these were involved in organ development (GO: 0048513) and response to mechanical stimulus (GO: 0009612).

**DISCUSSION**

We found that P3 and P14 BMSC express numerous genes encoding for proteins involved in oligodendroglial development and myelination, astroglial differentiation, and neuronal proliferation and neurite formation. Other genes expressed by these cells play a role in nervous system development in addition to their functioning in other pathways. These findings may reflect the described ability of BMSC to differentiate across lineages and become neural cells. If BMSC possess such an ability it would greatly benefit their use in nervous tissue repair as grafted BMSC could replace lost and/or damaged neural cells. It was reported that BMSC can differentiate into neural cells *in vivo*\(^2\)\(^5\) or be induced to become one *in vitro*\(^2\)\(^0\)\(^0\)\(^3\)\(^7\)\(^1\)\(^4\)\(^4\)\(^5\), but the evidence is controversial\(^6\)\(^6\),\(^2\)\(^4\)\(^3\),\(^2\)\(^9\)\(^1\).

We found that BMSC express genes that encode for proteins involved in the development of tissue in general and in growth factor signalling. The expression of these genes may indicate the potential of BMSC for cell-based repair as the gene products could promote tissue formation and functioning\(^3\)\(^7\),\(^7\). We also found that BMSC express genes involved in cell death mechanisms. This could be benefited from as these genes can be targeted to improve survival of BMSC transplanted into the injured nervous tissue. It has been demonstrated that survival of BMSC is low after transplantation in for instance the damaged spinal cord\(^1\)\(^7\)\(^0\),\(^3\)\(^8\)\(^5\). Improved survival could enhance the overall repair efficacy of a BMSC transplant\(^3\)\(^2\)\(^2\),\(^3\)\(^4\),\(^4\)\(^7\).

Our functional data mining revealed that 9 overrepresented GO-classes in P3 BMSC (compared to P14 BMSC) were involved in developmental processes and cell proliferation. On the other hand, 1 overrepresented GO-class in P14 (compared to P3 BMSC) was involved in organ development and mechanical stimulus. Together, these findings suggest a diminished plasticity of BMSC after long-term culturing. This idea is corroborated by the finding that the expression level of the SDF-1 gene was more than 3 times higher in P3 than P14 BMSC. SDF-1 gene encodes for a protein involved in brain development, motor axon guidance, and neuron migration\(^8\)\(^6\),\(^8\)\(^9\),\(^2\)\(^9\)\(^9\),\(^3\)\(^9\) and may be indicative of the overrepresentation of developmental processes in P3 compared to P14 BMSC. Also supporting the idea is that no genes involved in neural development were higher expressed in P14 BMSC than in P3 BMSC.

In the present study, the gene expression profiles of BMSC that were cultured for a shorter and longer period were compared to examine possible effects after long-term culturing. To our knowledge the effect of long-term culturing on gene expression in BMSC has not been investigated previously. In 3% of the genes that were expressed in P3 and in P14 BMSC,
expression levels had significantly changed after long-term culturing. Previously, it was shown that, after a sciatic nerve crush, the expression level of 5% of the genes in neurons that had their axon damaged changed significantly\cite{88}. This resulted in a crucial phenotypic change as this group of genes were involved in regenerative events in the damaged neurons\cite{88}. Franssen and colleagues\cite{39} showed that 7% of the genes that were differentially expressed between cultured Schwann cells and cultured olfactory ensheathing glial cells were expressed with more than 3 times difference. Further studies will be necessary to elucidate the relevance of the 3% of genes differentially expressed between P3 and P14 BMSC.

Our data showed that both P3 and P14 BMSC express genes involved in developmental and differentiation pathways in general, and in nervous system development, myelination and gliogenesis in particular. The overrepresentation of differently expressed genes involved in these pathways alters significantly between P3 and P14 and pointed at a decrease in plasticity in long-term cultured BMSC.
Gene expression pattern and suitability of bone marrow stromal cells

APPENDIX

Table 1. Genes expressed in P3 BMSC involved in nervous system pathways.

A: Nervous system development

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
</tr>
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<tbody>
<tr>
<td>NM_012987</td>
<td>7.31</td>
<td>Nes</td>
<td>Nestin</td>
<td>CNS development</td>
</tr>
<tr>
<td>XM_239373</td>
<td>7.69</td>
<td>Cog1</td>
<td>Predicted: component of oligomeric golgi complex 1</td>
<td>CNS development, myelin formation</td>
</tr>
<tr>
<td>NM_019139</td>
<td>11.69</td>
<td>Gdnf</td>
<td>Glial cell line derived neurotrophic factor</td>
<td>anti-apoptosis, cell proliferation, CNS/PNS, neurite development</td>
</tr>
<tr>
<td>L15011</td>
<td>7.56</td>
<td>Cun</td>
<td>Neuron-specific cortexin</td>
<td>CNS development</td>
</tr>
<tr>
<td>NM_012137</td>
<td>6.89</td>
<td>Cln2</td>
<td>Ceroid lipofuscinosis, neuronal 2</td>
<td>CNS development</td>
</tr>
<tr>
<td>NM_178866</td>
<td>6.92</td>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
<td>CNS development</td>
</tr>
<tr>
<td>NM_017003</td>
<td>9.15</td>
<td>Erbb2</td>
<td>V-erb-b2 erythroblast leukemia viral oncogene hom 2</td>
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</tr>
<tr>
<td>NM_1004401</td>
<td>7.74</td>
<td>Enc1</td>
<td>Ectodermal-neural cortex 1</td>
<td>CNS development</td>
</tr>
<tr>
<td>NM_1004231</td>
<td>10.32</td>
<td>Npdc1</td>
<td>neural prol / diff and control, 1</td>
<td>neural proliferation and differentiation</td>
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<tr>
<td>NM_012529</td>
<td>7.15</td>
<td>Ckb</td>
<td>creatine kinase, brain</td>
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B: Oligodendroglial development

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<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
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<tbody>
<tr>
<td>XM_239373</td>
<td>7.69</td>
<td>Cog1</td>
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<td>structural component of myelin sheath</td>
</tr>
<tr>
<td>NM_017003</td>
<td>9.147</td>
<td>Erbb2</td>
<td>V-erb-b2 erythroblast leukemia viral oncogene homolog 2</td>
<td>myelination</td>
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<tr>
<td>NM_012720</td>
<td>7.697</td>
<td>Mobp</td>
<td>Myelin-associated oligodendrocyte basic protein</td>
<td>myelin formation</td>
</tr>
<tr>
<td>NM_207601</td>
<td>12.61</td>
<td>Plp2</td>
<td>Proteolipid protein 2</td>
<td>myelin protein</td>
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C: Astroglial differentiation

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<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
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<tr>
<td>NM_012022</td>
<td>9.65</td>
<td>Cspg4</td>
<td>Chondroitin sulfate proteoglycan 4</td>
<td>glial cell migration, neuron fate commitment, remodelling, etc</td>
</tr>
<tr>
<td>NM_178866</td>
<td>6.92</td>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
<td>glial cell differentiation</td>
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<tr>
<td>XM_214090</td>
<td>9.72</td>
<td>Pdgfra</td>
<td>Predicted: platelet derived growth factor receptor, alpha polypeptide</td>
<td>gliogenesis</td>
</tr>
<tr>
<td>NM_017003</td>
<td>9.15</td>
<td>Erbb2</td>
<td>V-erb-b2 erythroblast leukemia viral oncogene homolog 2</td>
<td>glial cell differentiation</td>
</tr>
</tbody>
</table>
### D: neuronal processes

<table>
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<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
</tr>
</thead>
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<tr>
<td>NM_019139</td>
<td>11.69</td>
<td>Gdnf</td>
<td>Glial cell line derived neurotrophic factor</td>
<td>anti-apoptosis, cell proliferation, CNS/PNS, neurite development</td>
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<td>Ras homolog enriched in brain</td>
<td>regulation of neuronal synaptic plasticity</td>
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<tr>
<td>NM_012922</td>
<td>7.65</td>
<td>Casp3</td>
<td>Caspase 3</td>
<td>neuron apoptosis, cell fate commitment</td>
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<tr>
<td>NM_031022</td>
<td>9.64</td>
<td>Cspg4</td>
<td>Chondroitin sulfate proteoglycan</td>
<td>glial cell migration, neuron fate commitment, remodelling, etc</td>
</tr>
<tr>
<td>NM_012987</td>
<td>7.31</td>
<td>Nes</td>
<td>Nestin</td>
<td>neuron differentiation, CNS development</td>
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<tr>
<td>NM_130740</td>
<td>7.63</td>
<td>Pacsin2</td>
<td>Protein kinase C and casein kinase substrate in neurons 2</td>
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<tr>
<td>NM_053389</td>
<td>7.09</td>
<td>Sip1</td>
<td>Survival of motor neuron protein interacting protein 1</td>
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</tr>
<tr>
<td>NM_1025400</td>
<td>7.01</td>
<td>Smndc1</td>
<td>Predicted: survival motor neuron domain containing 1</td>
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<tr>
<td>XM_233798</td>
<td>10.76</td>
<td>Crim1</td>
<td>Predicted: cysteine-rich motor neuron 1</td>
<td></td>
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<tr>
<td>NM_031357</td>
<td>6.89</td>
<td>Cln2</td>
<td>Ceroid lipofuscinosis, neuronal 2</td>
<td>CNS development</td>
</tr>
<tr>
<td>L15011</td>
<td>7.55</td>
<td>Ctn</td>
<td>Neuron-specific cortexin</td>
<td>CNS development</td>
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Table 2. Expression of genes involved in cell cycle and developmental processes in P3 BMSC.

<table>
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<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
</tr>
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<tbody>
<tr>
<td>NM_178866</td>
<td>6.92</td>
<td>Igh1</td>
<td>Insulin-like growth factor 1</td>
<td>growth factor activity, anti-apoptosis, cell development</td>
</tr>
<tr>
<td>NM_013174</td>
<td>9.51</td>
<td>Tgfb3</td>
<td>Transforming growth factor, beta 3</td>
<td>protein binding, cell growth, embryonic development</td>
</tr>
<tr>
<td>XM_237999</td>
<td>11.51</td>
<td>Gadd45g</td>
<td>Predicted: growth arrest and DNA-damage-inducible 45 gamma</td>
<td>protein binding, cell differentiation, apoptosis, regulation of cell cycle</td>
</tr>
<tr>
<td>NM_053653</td>
<td>8.68</td>
<td>Vegfc</td>
<td>Vascular endothelial growth factor C</td>
<td>growth factor activity, cell proliferation, angiogenesis</td>
</tr>
<tr>
<td>NM_031022</td>
<td>9.65</td>
<td>Cspg4</td>
<td>Chondroitin sulfate proteoglycan 4 (Cspg4)</td>
<td>cell differentiation, cell proliferation, angiogenesis</td>
</tr>
<tr>
<td>XM_215993</td>
<td>8.74</td>
<td>Edfr</td>
<td>Predicted: endothelial differentiation-related factor 1</td>
<td>transcription, cell differentiation</td>
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<tr>
<td>XM_341934</td>
<td>7.56</td>
<td>Tgfb1i</td>
<td>Predicted: transforming growth factor beta 1 induced transcript 1</td>
<td>protein binding, cell differentiation, cell fate commitment</td>
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<tr>
<td>AB108787</td>
<td>9.03</td>
<td>Scf mRNA for stem cell factor KL-2, complete cds</td>
<td>stem cell factor receptor, regulation of apoptosis</td>
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<tr>
<td>XM_345649</td>
<td>7.53</td>
<td>Similar to development- and differentiation-enhancing factor 2; PYK2</td>
<td></td>
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<tr>
<td>XM_216438</td>
<td>7.43</td>
<td>ADRP</td>
<td>Adipose differentiation-related protein (ADRP)</td>
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<tr>
<td>XM_224733</td>
<td>7.01</td>
<td>Gdf1</td>
<td>Predicted: growth differentiation factor 1</td>
<td>growth factor activity</td>
</tr>
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</table>
Table 3. Expression of genes encoding for growth factors or proteins involved in growth pathways in P3 BMSC.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
</tr>
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<tbody>
<tr>
<td>NM_019139</td>
<td>11.68</td>
<td>Gdnf</td>
<td>Glial cell line derived neurotrophic factor</td>
<td>anti-apoptosis, cell proliferation, CNS/PNS, neurite development</td>
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<tr>
<td>NM_139259</td>
<td>7.83</td>
<td>Nradd</td>
<td>Neurotrophin receptor associated death domain</td>
<td>signal transduction, neurotrophin p75 binding</td>
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<td>NM_053401</td>
<td>9.64</td>
<td>Ngfrap1</td>
<td>Nerve growth factor receptor associated protein 1</td>
<td>induction of apoptosis</td>
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<tr>
<td>NM_053653</td>
<td>8.68</td>
<td>Vegfc</td>
<td>Vascular endothelial growth factor C</td>
<td>growth factor activity, cell proliferation, angiogenesis</td>
</tr>
<tr>
<td>NM_012801</td>
<td>8.59</td>
<td>Pdgfa</td>
<td>Platelet derived growth factor, alpha</td>
<td>growth factor activity, cell proliferation, cell migration</td>
</tr>
<tr>
<td>NM_1011921</td>
<td>10.35</td>
<td>Pdgfrl</td>
<td>Platelet derived growth factor receptor like</td>
<td>cell surface protein</td>
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<tr>
<td>XM_214030</td>
<td>9.72</td>
<td>Pdgfra</td>
<td>Predicted: platelet derived growth factor receptor, alpha polypeptide</td>
<td>gliogenesis, positive regulation of cell proliferation and cell migration</td>
</tr>
<tr>
<td>NM_031525</td>
<td>9.14</td>
<td>Pdgfra</td>
<td>Platelet derived growth factor receptor, beta polypeptid</td>
<td>anti-apoptosis, positive regulation of cell proliferation</td>
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<tr>
<td>NM_199114</td>
<td>8.25</td>
<td>Fgfr1</td>
<td>Fibroblast growth factor receptor-like 1</td>
<td>negative regulation of cell proliferation</td>
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<td>NM_022182</td>
<td>9.23</td>
<td>Fgf7</td>
<td>Fibroblast growth factor 7</td>
<td>growth factor activity, positive regulation of cell proliferation</td>
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<tr>
<td>NM_178866</td>
<td>6.92</td>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
<td>anti-apoptosis, cell development, glial cell differentiation</td>
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<td>NM_1004274</td>
<td>10.41</td>
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<td>Insulin-like growth factor binding protein 4</td>
<td>regulation of cell growth</td>
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Table 4. Expression of genes involved in cell death mechanisms in P3 BMSC.

<table>
<thead>
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<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
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<tbody>
<tr>
<td>XM_226833</td>
<td>8.29</td>
<td>C1qtnf3</td>
<td><em>Predicted:</em> C1q and tumor necrosis factor related protein 3</td>
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<tr>
<td>NM_139194</td>
<td>8.07</td>
<td>Tnfrsf6</td>
<td>Tumor necrosis factor receptor superfamily, member 6</td>
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<td>Tnfrsf4</td>
<td>Tumor necrosis factor receptor superfamily, member 4</td>
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<td>NM_01006952</td>
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<td>Trap1</td>
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<td>Tnfrsf1a</td>
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<td>Type 1 tnf receptor shedding aminopeptidase regulator</td>
<td>positive regulation of angiogenesis</td>
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<tr>
<td>NM_181086</td>
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<td>Tnfrsf12a</td>
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<td>cell death</td>
</tr>
<tr>
<td>BC061751</td>
<td>7.96</td>
<td></td>
<td>Androgen receptor-related apoptosis-assoc protein CBL27</td>
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<tr>
<td>XM_342470</td>
<td>9.91</td>
<td>Apis5</td>
<td><em>Predicted:</em> apoptosis inhibitor 5</td>
<td>anti-apoptosis</td>
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<tr>
<td>XM_216650</td>
<td>9.14</td>
<td></td>
<td><em>Predicted:</em> similar to apoptosis rel protein APR-3; p18 protein</td>
<td></td>
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<tr>
<td>NM_053516</td>
<td>9.09</td>
<td>Nol3</td>
<td>Nucleolar protein 3 (apoptosis repressor with CARD domain)</td>
<td>regulation of apoptosis</td>
</tr>
<tr>
<td>XM_342137</td>
<td>8.45</td>
<td>Amid</td>
<td><em>Predicted:</em> apoptosis-inducing factor (AIF)-like mitochondrial induction of death</td>
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<tr>
<td>XM_235661</td>
<td>8.34</td>
<td></td>
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<td>NM_053736</td>
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<td>Casp11</td>
<td>Caspase 11</td>
<td>regulation of apoptosis, inflammatory response</td>
</tr>
<tr>
<td>NM_022522</td>
<td>7.69</td>
<td>Casp2</td>
<td>Caspase 2</td>
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<tr>
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<td>Casp3</td>
<td>Caspase 3</td>
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<td>7.291</td>
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<td>Entrez ID</td>
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<td>Function</td>
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<tr>
<td>NM_139259</td>
<td>7,827</td>
<td><strong>Nradd</strong> Neurotrophin receptor associated death domain</td>
<td>signal transduction, apoptosis</td>
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<tr>
<td>NM_019339</td>
<td>11,69</td>
<td><strong>Gdnf</strong> Glial cell line derived neurotrophic factor</td>
<td>anti-apoptosis, cell proliferation</td>
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</tr>
<tr>
<td>NM_199270</td>
<td>7,79</td>
<td><strong>Bre</strong> Brain and reproductive organ-expressed protein</td>
<td>apoptosis</td>
<td></td>
</tr>
<tr>
<td>NM_053401</td>
<td>9,64</td>
<td><strong>Ngfrap1</strong> Nerve growth factor receptor (TNFRSF16) associated protein 1</td>
<td>induction of apoptosis</td>
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</tr>
<tr>
<td>NM_133546</td>
<td>6,87</td>
<td><strong>Myd116</strong> Myeloid differentiation primary response gene 116</td>
<td>apoptosis, response to stress</td>
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</tr>
<tr>
<td>NM_012588</td>
<td>8,52</td>
<td><strong>Igfbp3</strong> Insulin-like growth factor binding protein 3</td>
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<tr>
<td>NM_01012154</td>
<td>7,179</td>
<td><strong>Tbrg4</strong> Predicted: Transforming growth factor beta regulated gene 4</td>
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<tr>
<td>XM_216265</td>
<td>6,842</td>
<td><strong>Ing4</strong> Predicted: Inhibitor of growth family, member 4</td>
<td>apoptosis, cell cycle arrest, neg</td>
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<tr>
<td>XM_237999</td>
<td>11,51</td>
<td><strong>Gadd45</strong> Predicted: Growth arrest and DNA-damage-inducible 45 gamma</td>
<td>apoptosis, regulation of cell cycle</td>
<td></td>
</tr>
<tr>
<td>NM_133544</td>
<td>11,04</td>
<td><strong>Usmg5</strong> Upregulated during skeletal muscle growth 5</td>
<td>apoptosis, regulation of cell cycle</td>
<td></td>
</tr>
<tr>
<td>NM_012756</td>
<td>10,76</td>
<td><strong>Igfr2</strong> Insulin-like growth factor 2 receptor</td>
<td>regulation of apoptosis</td>
<td></td>
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<tr>
<td>NM_013174</td>
<td>9,51</td>
<td><strong>Tgb3</strong> Transforming growth factor, beta 3</td>
<td>regulation of apoptosis, cell growth, regulation of cell cycle</td>
<td></td>
</tr>
<tr>
<td>NM_031525</td>
<td>9,14</td>
<td><strong>Pdgfrb</strong> Platelet derived growth factor receptor, beta polypeptide</td>
<td>anti-apoptosis, positive regulation of cell proliferation</td>
<td></td>
</tr>
<tr>
<td>NM_017003</td>
<td>9,15</td>
<td><strong>Erbb2</strong> V-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
<td>anti-apoptosis, positive regulation of cell proliferation</td>
<td></td>
</tr>
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</table>
Table 5. Differentially expressed genes between P3 and P14 BMSC with A > 3 fold difference.

A: Upregulated at P14

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_053021</td>
<td>3.96</td>
<td>Clu</td>
<td>Clusterin (Clu)</td>
<td>protein binding, apoptosis, cell differentiation /proliferation, etc</td>
</tr>
<tr>
<td>U87985</td>
<td>3.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_012862</td>
<td>3.39</td>
<td>Mgp</td>
<td>Matrix Gla protein</td>
<td>regulation of bone mineralisation</td>
</tr>
<tr>
<td>XM_57454</td>
<td>3.09</td>
<td>LOC49156</td>
<td><em>Predicted:</em> similar to growth arrest-specific protein 2 - mouse</td>
<td>cell cycle arrest</td>
</tr>
<tr>
<td>NM_0010118</td>
<td>3.04</td>
<td>Cldyn</td>
<td><em>Predicted:</em> claudin 9</td>
<td>cell adhesion molecule</td>
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</table>

B: Upregulated at P3

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Exp</th>
<th>Gene</th>
<th>Description</th>
<th>Ontology</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_012893</td>
<td>2.99</td>
<td>Actg2</td>
<td>Actin, gamma 2</td>
<td>protein binding, cytoskeleton</td>
</tr>
<tr>
<td>NM_080886</td>
<td>3.19</td>
<td>S4mol</td>
<td>Sterol-C4-methyl oxidase-like</td>
<td>oxidation reduction</td>
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<tr>
<td>XM_231258</td>
<td>3.25</td>
<td>E-FABP</td>
<td>similar to fatty acid-binding protein, epidermal</td>
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<tr>
<td>NM_021776</td>
<td>3.27</td>
<td>Gda</td>
<td>Guanine deaminase</td>
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</tr>
<tr>
<td>XM_233057</td>
<td>3.30</td>
<td>Pappa</td>
<td><em>Predicted:</em> pregnancy-associated plasma protein A</td>
<td></td>
</tr>
<tr>
<td>XM_214778</td>
<td>3.45</td>
<td>TSP-2</td>
<td>Thrombospondin 2</td>
<td>angiogenesis, positive regulation of synaptogenesis</td>
</tr>
<tr>
<td>XM_573502</td>
<td>3.78</td>
<td></td>
<td><em>Similar:</em> Fc gamma (IgG) receptor II alpha precursor</td>
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</tr>
<tr>
<td>AF217564</td>
<td>3.79</td>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1 gamma</td>
<td>brain development, motor axon guidance, neuron migration, etc</td>
</tr>
<tr>
<td>NM_1007612</td>
<td>3.87</td>
<td>Ccl7</td>
<td>Chemokine (C-C motif) ligand 7</td>
<td>chemokine activity, immune response</td>
</tr>
<tr>
<td>NM_030834</td>
<td>4.40</td>
<td>Slc16a3</td>
<td>Monocarboxylate transporter</td>
<td>transporter activity</td>
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<tr>
<td>NM_031350</td>
<td>4.44</td>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>anti-apoptosis, immune response, chemokine activity</td>
</tr>
<tr>
<td>NM_019282</td>
<td>5.09</td>
<td>Greml1</td>
<td>Gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)</td>
<td>apoptosis, cell-cell signalling</td>
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</tbody>
</table>
Table 6. Overrepresented GO-classes in significantly different expressed genes between P3 and P14 BMSC (A > 1.5 fold difference) compared to total subset of expressed genes.

<table>
<thead>
<tr>
<th>GO CLASS</th>
<th>GENE ONTOLOGY</th>
<th>GROUP</th>
<th>Total</th>
<th>P-value</th>
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<td>Biological process</td>
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<td></td>
<td></td>
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<td>GO:0048513</td>
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<td>252</td>
<td>3.45E-06</td>
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<td>alcoholic metabolic process</td>
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<td>75</td>
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<tr>
<td>GO:0008202</td>
<td>steroid metabolic process</td>
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<td>37</td>
<td>4.10E-06</td>
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<tr>
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<td>554</td>
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<td>409</td>
<td>6.82E-05</td>
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<td>cholesterol metabolic process</td>
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<td>17</td>
<td>6.82E-05</td>
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<td>GO:0048731</td>
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<td>GO:0009012</td>
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<td>GO:0006695</td>
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<td>GO:0009058</td>
<td>biosynthetic process</td>
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<td>325</td>
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<td>GO:0030190</td>
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<td>4</td>
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<td>GO:0044255</td>
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<td>GO:0006935</td>
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<td>17</td>
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<tr>
<td>GO:00442330</td>
<td>taxis</td>
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<td>17</td>
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<td>GO:00091444</td>
<td>vasculature development</td>
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<td>glucose metabolic process</td>
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<td>di-, tri-valent inorganic cation homeostasis</td>
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<tr>
<td>GO:0050801</td>
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<td>55</td>
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**Cellular component**

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<tr>
<th>GO:0044421</th>
<th>extracellular region part</th>
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<th>306</th>
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**Molecular function**

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<th>GO:0008201</th>
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<td>chemokine activity</td>
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<td>pattern binding</td>
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<td>20</td>
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<td>GO:0005539</td>
<td>glycosaminoglycan binding</td>
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**UPREGULATED AT P<14**

<table>
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<th>GO:0008313</th>
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<tr>
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<td>response to mechanical stimulus</td>
<td>3</td>
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<td>0.008</td>
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</table>

**Cellular component**

| GO:0005978 | proteinaceous extracellular matrix | 6 | 59 | 0.004 |
Survival and neuroprotection.

Bone marrow stromal cells elicit tissue sparing after acute but not delayed transplantation into the contused adult rat thoracic spinal cord.

*J Neurotrauma* 2009; 26 (12): 2313-2322

R.D.S. Nandoe Tewarie
A. Hurtado
G.J. Ritfeld
S.T. Rahiem
D.F. Wendel
M. M. S. Barroso
J. A. Grotenhuis
M. Oudega
INTRODUCTION

A contusion of the adult spinal cord causes acute death of neural cells at the lesion epicenter and sets off a series of events resulting in additional tissue loss and the formation of cystic cavities. Transplantation of growth-promoting cells is considered a promising approach for repair of the contused spinal cord.

Bone marrow stromal cells (BMSC) are among the candidates for cell-based spinal cord repair strategies. These mesenchymal cells secrete growth factors that could support repair. Their potential was confirmed by the finding that transplantation of BMSC into the contused adult rat spinal cord resulted in functional recovery. The mechanisms underlying these functional improvements observed after BMSC transplantation are incompletely understood.

A BMSC graft could contribute to repair by sparing spinal cord nervous tissue at the site of transplantation. It is known that BMSC secrete brain-derived neurotrophic factor (BDNF), which has been implicated in limiting spinal cord tissue loss after injury. At present, the evidence for BMSC grafts to elicit tissue sparing in the injured spinal cord is still ambiguous.

Following an insult to the spinal cord, a cytotoxic environment develops at the injury epicenter to diminish in strength in time. Most likely these circumstances decrease the survival of cells transplanted into the lesion and consequently their effects on spinal cord repair. At present, quantitative evidence of the survival and neuroprotective effects of BMSC transplanted into a spinal cord contusion is sparse, which obscures their spinal cord repair potential. We transplanted BMSC into a moderately contused adult rat spinal cord at 15 min (acutely) and at 3, 7 and 21 days (delayed) post-injury and determined tissue sparing and BMSC survival up to 4 weeks post-transplantation.

MATERIAL AND METHODS

Timeline of the experiment

Rats received a moderate contusion of the T9 spinal cord. Some of these rats received a BMSC injection into the injury epicenter at 15 min, 3, 7, or 21 days post-contusion. These BMSC-transplanted rats were euthanized at 15 min, 3, 7, and 28 days post-injection. Other contused rats did not receive the BMSC injection (control rats) and, to match survival times of the BMSC-transplanted rats, were euthanized at 15 min, and 3, 7, 10, 14, 21, 24, 28, 31, 35, and 49 days post-injury. Their spinal cords were removed and prepared for histology to enable analysis of tissue sparing and BMSC numbers. Details on the used techniques are described below.
Animals
Adult female Sprague-Dawley rats (n = 121), 160-180 g; Harlan, Indianapolis, IN, USA) were used in these experiments. All animals were housed according to the guidelines by the National Institutes of Health and the United States Department of Agriculture. The institutional Animal Care and Use Committees of the University of Miami and Johns Hopkins University approved all surgical procedures.

Culture and lentiviral transduction of BMSC
BMSC were obtained from the marrow of femurs and tibias of adult female Sprague-Dawley rats (n = 8) according to a previously published protocol. Passage 0 cells were infected with lentiviral vectors (LV) encoding for green fluorescent protein (GFP) at an MOI of 150. The vectors were generated using the ViraPower Lentiviral Expression System (Invitrogen). Expression was under control of the human cytomegalovirus promoter and the Woodstuck hepatitis virus Post-transcriptional Regulatory Element. The titer of the lentiviral stocks varied from 1-3 x 10⁹ TU/ml. Only passage 3 cultures with a transduction rate of about 95% were used for transplantation.

With enzyme-linked immune sorbent assays (ELISA) we determined whether the BMSC secreted BDNF and glial cell-derived neurotrophic factor (GDNF). For this, medium of nearly confluent passage 3 cultures (with an average of 2 x 10⁶ cells) was refreshed, removed 24 h later, and used for BDNF ELISA (BDNF Emax ImmunoAssay System; Promega Corporation, Madison, WI) and GDNF ELISA (GDNF Emax ImmunoAssay System; Promega) according to the manufacturer’s instructions. Standard curves of the ELISA kits were linear between 7.8 and 500 pg/ml for BDNF and 15.6 and 1000 pg/ml for GDNF. ELISAs were analyzed with a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). The cells in culture secreted 14.1 pg/ml/24 h of BDNF. The amount of GDNF was below the reliability level of the ELISA.

Contusion injury
Rats (n = 108) were anesthetized with 1-2% isoflurane in oxygen and their backs were shaved and cleaned with Betadine and 70% alcohol. Lacrilube ophthalmic ointment (Allergen Pharmaceuticals, Irvine, CA, USA) was applied to the eyes and gentamicin (1.2 mg in 0.03 ml; Abbott Laboratories, North Chicago, IL, USA) was injected intramuscularly. During surgery, rats were kept on a heating pad to maintain their body temperature at 37 ± 0.5 °C. A laminectomy was performed at the eighth thoracic vertebra to expose the ninth thoracic spinal cord segment, which was subsequently moderately contused (NYU impactor; 10 g, 12.5 mm.; Gruner 1992). To ensure consistency between contused rats, the contusion impact velocity and compression were monitored and all rats with more than 5% error in these measurements were removed from the study. Rats that remained in the study displayed
hindlimb paralysis and scored less than 3 at 1 day and less than 7 at 3 days post-injury on the Basso-Beattie-Bresnahan (BBB) scale\textsuperscript{23, 24}. Implementation of these criteria ensured appropriateness of the contusion injury and consistency among the experimental animals. All laminectomies and injuries were performed by the same investigator. After injury, overlying muscles were sutured in layers and the skin was closed with metal wound clips. The rats received Ringer's solution (10 ml, subcutaneous) and gentamicin (1.2 mg, intramuscular). The rats were kept in a small animal incubator at 37 °C until full recovery and then returned to their cages with ad libitum access to water and food. During the first week post-injury gentamicin (1.2 mg; Abbott Laboratories, North Chicago, IL, USA; intramuscular) was administered daily. The analgesic, buprenorphine (Buprenex\textsuperscript{®}; 0.006 mg; Reckitt Benckiser, Richmond, VA, USA; subcutaneous) was administered immediately after surgery and once for the next three days. The bladders were expressed manually twice a day until reflex bladder function returned. Urinary tract infection did not occur in any of the groups.

**BMSC transplantation**

Contused rats were anaesthetized with an intramuscular injection of 25.7 mg/kg ketamine, 5.14 mg/kg xylazine, and 0.85 mg/kg acepromazine and their contused spinal cord was exposed. A total of $1 \times 10^6$ BMSC in 5 μl DMEM was injected into the contusion epicenter using techniques described previously\textsuperscript{175}. BMSC injections were made at 15 min, 3, 7, and 21 days post-contusion. In the 15 min group, the rats remained sedated with the contused spinal cord covered with a saline-moisten gauze until BMSC injection. All transplantations were performed by the same investigator. After injection, muscles were sutured in layers and the skin closed with metal wound clips. Post-surgery care was as described above.

**Assessment of BMSC viability during the injection procedures**

The effects of the injection procedures on the number of BMSC were determined in vitro using the same tools and methods as used for actual transplantation. First, we assessed the viability of $1 \times 10^6$ BMSC in 5 μl DMEM in an eppendorf tube after 5 h on ice (the typical length of a transplantation session). A sample of these cells was stained with trypan blue (1:1; Sigma) and the percentage of living (unstained) BMSC calculated using a hemocytometer. Second, we assessed BMSC viability after injection. BMSC in DMEM were kept on ice for 5 h and then injected into an eppendorf tube ($1 \times 10^6$ BMSC in 5 μl DMEM per tube) using the same pulled glass injection needle attached to a Hamilton syringe as used for transplantation into the rat spinal cord. These cells were then kept at 37 °C/5% CO$_2$ for 1, 3, and 5 h (n = 3 for each time point). BMSC that were not passed through the glass needle and kept in the incubator for the same time periods served as controls (n = 3 for each time point). At the selected times, and immediately after passing through the glass needle (time point 0), cells were stained with trypan blue (1:1; Sigma) and the percentage of living (unstained) BMSC calculated using a
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The effect of passing through the glass needle was determined by the difference in the percentages of living BMSC between control and time point 0 cells. Whether the incubation conditions had affected BMSC death in time was calculated from the difference in viability between control cells and cells that were passed through the glass needle at 1, 3, and 5 h. Third, we determined how many cells passed through the needle. A sample of $1 \times 10^6$ BMSC in $5 \mu$l DMEM that were injected into an eppendorf tube using a pulled glass needle attached to a Hamilton syringe was stained with trypan blue (1:1; Sigma) to calculate the total number of living ( unstained) BMSC using a hemocytometer.

**Histological procedures**

At 15 min, 3, 7, and 28 days after BMSC injection, rats were anaesthetized as above (note that the 15 min group remained anaesthetized until fixation). Control rats that were contused but did not receive a BMSC transplant were anaesthetized 15 min, and 3, 7, 10, 14, 21, 24, 28, 31, 35, and 49 days post-injury. These time points correspond with the survival times of the BMSC-transplanted rats. After deep sedation was confirmed, the heart was exposed and 0.1 ml Heparine (500 IU; Henry Schein, Melville, NY, USA) injected into the left ventricle. Next, 500 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) was pumped through the vascular system. The spinal cord was removed and kept overnight in the same fixative at 4 °C. Then, a 15 mm long segment of the spinal cord centered at the contusion was dissected out, kept in phosphate-buffered 30% sucrose (0.1 M, pH 7.4) for 48 h, and frozen within Shandon M-1 Embedding Matrix (Thermo Electron Corporation, Pittsburgh, PA, USA). From these blocks, 20 µm-thick horizontal sections were cut on a cryostat, mounted onto glass slides, and stored at -20 °C.

From some rats of each group, a 1 mm thick section was taken from the epicenter of the lesion for preparation of semithin plastic sections for light microscopy. The 1 mm thick tissue blocks were kept in 2% glutaraldehyde with 3% sucrose in PB for at least 24 h at 4 °C, in 1% osmium tetroxide in PB for 12-16 h, dehydrated, and embedded in Epon-Araldite (Electron Microscopy Services, Fort Washington, PA). One-mm-thick transverse sections were cut on a Reichert-Jung ultra-microtome, stained with a 1% toluidine blue-1% methylene blue-1% sodium borate solution and used to analyze general histology of the spinal cord and transplant and determine tissue loss at the injury/transplant epicenter.

**Measurement of spinal cord tissue sparing**

Sparing of spinal cord tissue was assessed in a blinded fashion in horizontal cresyl violet-stained cryostat sections using the Cavalieri estimator function of Stereo Investigator® (MBF Bioscience, Williston, VT). All measurements were taken by the same investigator. From each animal, every tenth cryostat section (200 µm intervals) was used to determine the volume of a 5 mm long segment centered at the contusion/transplantation epicenter and that of spared
tissue within this segment. Tissue was considered spared if it lacked cavities, areas with high density of infiltrating small cells, resembling neutrophils and lymphocytes, and neurons with darkly-stained cytoplasmic Nissl bodies\textsuperscript{409}. The volume of spared tissue was expressed as a percentage of the average volume of a comparable uninjured spinal cord segment.

**Measurement of the number of BMSC within the contused spinal cord**

From each rat transplanted with BMSC, every tenth cryostat section was covered with a glass slip with Vectashield with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Inc.) and used to determine the number of GFP-positive BMSC in the contusion. In these sections, in a blinded fashion, the contusion area with GFP-positive cells was outlined under a 2.5 x objective. Then, under a 63 x objective (oil immersion), grids of 250 by 250 μm (counting frame area, 625 μm\(^2\)) were placed over the outlined area and each GFP-positive cell with a recognizable nucleus was counted using the optical fractionator function of Stereo Investigator\textsuperscript{®} (MBF Bioscience, Williston, VT, USA). The sampling interval was: \(x = 250 \, \mu m, \ y = 250 \, \mu m\). All measurements were taken by the same investigator. The resulting numbers were used to calculate the total number of GFP-positive BMSC within the contusion. These total numbers were then expressed as a percentage of the total number found at 15 min post-injection.

**Assessment of BMSC migration**

We found that transplanted BMSC had migrated away from the injection site. Their number, location, and distance from the contusion epicenter were determined in one series of cryostat sections of each of the transplanted rats. Because it is possible that BMSC had migrated beyond 8 mm, which was the approximate distance from the injury epicenter to the edge of the section in rostral and caudal direction, we also sectioned and examined the contiguous 10-mm long rostral and caudal spinal cord segment. All GFP-positive BMSC outside of the contusion were counted manually under a 40 x objective.

**Statistical analysis**

We used Sigmastat\textsuperscript{®} (Systat Software, Inc., San Jose, CA, USA) for the statistical analyses. The t-test was used to determine differences between groups which were accepted at \(p < 0.05\). The relationship between tissue sparing and BMSC number was determined by the Pearson correlation coefficient \(r\).
RESULTS

**BMSC transplants in the contused spinal cord**

With all transplantation paradigms, GFP-positive BMSC were found within the contusion at 15 min after the injection (Figs. 1A-C). The number of BMSC within the contusion was noticeably decreased at 7 days (Figs. 1D-F) and 28 days (Figs. 1G-I) after injection.

![Image of BMSC transplants](image)

**Spared tissue in the contused adult rat spinal cord**

We qualitatively assessed the contused spinal cord segment of adult rats without (controls) and with a BMSC transplant using toluidine blue-stained transverse semithin plastic sections of the contusion epicenter and cresyl violet-stained horizontal cryostat sections of the contused spinal cord segment (Fig. 2). In transverse plastic sections, a loosely organized transplant that filled up the contusion was found at 3 days after an acute injection (Fig. 2A). At 28 days after a BMSC injection into the 21-day old contusion the spinal cord was not only decreased in size but also contained one or more large cavities (Fig. 2B). On horizontal sections, after an acute injection, cavities were not present at 3 days (Fig. 2C) but could be
found at 28 days (Fig. 2D). With a 7 day delayed injection, small cavities were found at 3 days (Fig. 2E) and much larger ones at 28 days (Fig. 2F) post-injection.

Fig. 2. Spared tissue in the contused adult rat spinal cord after BMSC transplantation. (A) Loosely organized tissue in contusion epicenter at 3 days after an acute BMSC injection. (B) Cavities present in the contusion epicenter at 28 days after a 21 day delayed transplantation of BMSC. Note that the spinal cord has also decreased in size. (C) At 3 days after acute BMSC injection, damaged tissue but not cavities was observed. (D) At 28 days after acute injection, large cavities were found in the contused segment. (E) With a 7 day delayed BMSC injection, damaged tissue and few small cavities were found at 3 days post-transplantation. (F) At 28 days after BMSC injection into the 7-day old contusion, large cavities were found in the contused segment. Images in A and B are from toluidine blue-stained transverse semithin plastic sections. Images in C-F are from cresyl violet-stained horizontal cryostat sections (rostral to the left). Bar in A represents 150 μm in (A-B). Bar in C represents 600 μm in (C-F).

Using Stereo Investigator™ software (MBF Bioscience) we determined that the volume of spared tissue in the contused spinal cord segment gradually decreased to 10.1 ± 1.7 mm³ at 49 days post-injury in control rats and to 11.5 ± 0.9 mm³ at 49 days post-injury in BMSC transplanted rats (Table 1). Because narrowing of the contused segment occurs, we determined the volume of spared tissue relative to that of a comparable segment from uninjured and untreated spinal cord (21.9 ± 3.4 mm³; SD, n = 5; Fig. 3A). We found that with BMSC injected at 15 min, 3, or 7 days after injury the volume of spared tissue was significantly higher in transplanted rats than in control rats at the respective endpoints (i.e., 28, 31, and 35 days post-injury).

We then determined the relative change in spared tissue volume in transplanted and control rats between the same time periods after contusion (Fig. 3B). Importantly, in BMSC
transplanted rats the volume of spared tissue at 15 min after injection was similar to that found in control rats of matching survival times. The results show that tissue sparing during the 28-days that BMSC were present was increased with acute and 3-days delayed but not with 7 and 21-days delayed BMSC transplantation compared to the loss that occurs in control rats within the same time periods.

**Fig. 3.** BMSC transplantation elicits tissue sparing in the contused adult rat spinal cord. (A) Bar graph showing the volumes of spared tissue in the contused spinal cord segment in rats without (control, open bars) and with (closed bars) BMSC transplants. Spared tissue volume is expressed as a percentage of the volume of a comparable uninjured spinal cord segment. Time points of the x-axis refer to control rats. The survival times of BMSC-transplanted rats that matched these time points were 15 m/15 m, 15 m/3 d, 15 m/7 d, 3 d/7 d, 7 d/7 d, 21 d/15 m, 21 d/3 d, 15 m/28 d, 3 d/28 d, 7 d/28 d, and 21 d/28 d (see also Table 1). Asterisks indicate a significant difference control and BMSC transplanted rats (* = p<0.05; ** = p<0.03; *** = p<0.01). (B) In this graph the decrease in spared tissue volume occurring during the 28 day time period is shown. The volume at the endpoint is expressed as a percentage of the volume at the starting point. The time periods refer to control rats. The matching time points of transplanted rats are listed above.
Loss of BMSC transplanted into the moderately contused rat spinal cord

We found that 95% of BMSC survived a period of 5 h on ice and 92% survived when passed through a pulled glass needle. Thus, our methods could have resulted in a maximum of 13% death among BMSC injected into the contusion. From the intended 1 million cells, $6.7 \times 10^5$ BMSC passed through the needle alive. Taking the greatest possible percentage of death due to transplantation into account, this implies that $8 \times 10^5$ BMSC were injected into the contusion of which $1.3 \times 10^4$ cells (13%) were dead. To correct for variability due to our procedures, BMSC numbers were expressed as a percentage of the number found at 15 min post-injection.

We found at 15 min post-injection that 35%, 25%, 58%, and 32% of the calculated average of $6.7 \times 10^5$ (live) BMSC were present and alive in the contusion with the injection made at 15 min, 3, 7 and 21-days post-injury, respectively. There was no direct relationship between the percentages and the timing of the injection; cell loss during the first 15 min post-injection was 35% with acute and 38% with delayed paradigms.

The average numbers of BMSC and corresponding percentages (relative to the number at 15 min post-injection) are listed in Table 2 (also Fig. 4A) and reveal that the number of BMSC at 7 days post-injection was significantly higher after acute ($p < 0.01$) and 3-days delayed ($p < 0.005$) injections compared to 7-days and 21-days delayed injections; 32% and 52% vs. 9% and 9%, respectively (Fig. 4B). The number of BMSC in the contusion at 28 days post-injection was close to 0 in all groups, indicating rejection of the transplanted cells.

Tissue sparing and BMSC survival are strongly associated

The relationship between tissue sparing and BMSC survival was assessed using the Pearson correlation coefficient. We found that with an acute and 3-days delayed injection, the positive correlation was large with $r = 1.0$ at 3, 7, and 28 days post-injection. With a 7-days delayed injection, the positive correlation was large with $r = 0.79$ at 3 days and with $r = 1.0$ at 7 and 28 days post-injection. With a 21-days delayed injection, there was no correlation between BMSC number and spared tissue volume. Thus, in groups that received the BMSC transplant during the first week post-contusion, we found a strong positive association between tissue sparing and BMSC survival with $r$ values between 0.79 and 1.0.
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Fig. 4. BMSC survival in a moderate contusion in the adult rat thoracic spinal cord is low. (A) The number of BMSC transplanted acutely (15 min post-injury) or delayed (3, 7, and 21 days post-injury) into a moderate contusion decreases over 28 days post-injection. The numbers are expressed as a percentage of the numbers found at 15 min post-injection. (B) BMSC death within the moderate contusion at 7 days post-injection is significantly lower after acute and 3-day delayed injections than with 7- and 21-day delayed injections. Percentages are relative to the number at 15 min post-transplantation. Bars represent standard error of the mean. * = p<0.01; ** = p<0.005.

Migration of BMSC into adjacent spinal nervous tissue

The number of BMSC in the transplant site could decrease as BMSC migrate away from the injection site. We found that a few days after injection BMSC started to migrate away mainly from the rostral and caudal aspects of the transplant (Figs. 5A). One week after injection, BMSC were found in the dorsal and ventral columns some as far as 7 mm away from the contusion epicenter. Most of these cells were spindle-shaped with a small nucleus and several extensions (Fig. 5B). With a 3 day post-injury injection, a total of 2,100 ± 849 BMSC at 7 days and 820 ± 99 at 28 days post-transplantation had migrated away from the transplant site (Fig. 5C). Significantly (p < 0.001) more BMSC were found rostral (80 ± 14%) than caudal (20 ± 9%) to the transplantation site. The total number of migrated BMSC represented 1.2% of the number of BMSC in the contusion at 15 min post-injection and 2.4% of the number at 7 days post-injection. At 28 days post-injection, almost twice as many BMSC were found outside of the contusion compared to within. Thus, BMSC that had migrated away from the injection site into the adjacent spinal cord tissue appeared to be protected compared to those that remained within the contusion.
Fig. 5. BMSC transplanted into the moderately contused adult rat spinal cord contusion migrate into spinal cord white matter. (A) GFP-positive BMSC (arrow) ‘leaving’ the transplant (T) at its rostral edge. (B) Several GFP-positive BMSC that have migrated approximately 5 mm into the rostral lateral white matter (WM). (C) Bar graph showing the number of BMSC that migrated out of a transplant at 7 and 28 days post injection. BMSC were injected at 3 days post-injury. All microphotographs confocal images from horizontal 20 μm thick cryostat sections. Bar in A represents 15 μm in panels A and B.

**DISCUSSION**

Survival of BMSC transplanted acutely (15 min post-injury) or delayed (3, 7, and 21 days post-injury) into a moderately contused adult rat spinal cord is low with few or none of the cells left at 28 days post-injection. BMSC loss at the 7-day post-injection time point was 68% with acute and 48% with 3-days delayed injections which was significantly lower than with 7- and 21-days (91% both) delayed injections. This indicated that the contusion environment is more deleterious to transplanted BMSC during the second and fourth week after impact than during the first week after impact. In control rats, the amount of spared tissue gradually decreased in time until 46% of that in a comparable uninjured spinal cord segment at 49 days post-injury. The presence of BMSC resulted in increased amounts of spared tissue compared to controls. Acute and 3-days delayed, but not 7- and 21-days delayed, injection of BMSC significantly improved tissue sparing. There was a large positive correlation between spared tissue volume and BMSC number with injections during the first week post-contusion.
Effects of BMSC on tissue sparing were assessed by determining the volume of spared (intact) tissue within the contused spinal cord segment. This revealed an increased volume of spared tissue at 7 and 28 days post-injury with an acute BMSC injection and at 6, 10, and 31 days post-injury with a 3-day delayed BMSC injection. This particular approach of measuring spared tissue is employed regularly but it does not take into account narrowing of the spinal cord, which typically occurs after a contusion. Therefore, we also determine the volume of spared tissue relative to that of a comparable segment from uninjured and untreated spinal cord. This revealed an increased volume of spared tissue at the abovementioned time points but also at 35 days post-injury with a 7-days delayed BMSC injection.

Our results demonstrated that acutely transplanted BMSC contributed to sparing of nervous tissue following a moderate contusion of the thoracic rat spinal cord better than delayed transplanted BMSC. The reduced loss of the acutely transplanted BMSC during the first week after injection correlated closely with the increased volumes of tissue sparing. Previously, it was reported that acutely transplanted BMSC reduced the size of cavities at 3 weeks after an injection into the contusion and at 5 weeks after an injection into the 4th ventricle. In the present study, we have assessed spared tissue volume relative to that of a comparable segment from an uninjured and untreated spinal cord to account for the normally occurring shrinkage. Despite this difference in approach, our results are in agreement as they indicated that acutely transplanted BMSC exert neuroprotective actions on the nervous tissue within the contused segment. The effects of these neuroprotective actions remain visible until 28 days post-transplantation. Yoshihara and collaborators demonstrated that transplantation of BMSC into a 9-day old moderate contusion did not result in neuroprotection within the contused segment which is in concurrence with our findings that delayed transplanted BMSC fail to exert neuroprotective actions. One potential mechanism by which transplanted BMSC result in neuroprotection may be by secreting BDNF. We used ELISA to confirm that the BMSC used for transplantation in our study produced and secreted BDNF. Future studies in which the production of BDNF by BMSC or the actions of BDNF are blocked will help to elucidate the role of BMSC-derived BDNF on neuroprotection.

Our data showed that 0.02% of acutely transplanted BMSC had survived up to 28 days in a moderate spinal cord contusion. In a previous study, it was shown that 0.17% of BMSC acutely injected into and near a moderate-severe contusion (25 mm, NYU impactor) in the rat spinal cord had survived at 5 weeks post-injury/injection. When BMSC were grafted into a 7-day old contusion, 6 times more cells (0.99%) had survived at 5 weeks post-injury (i.e., 4 weeks post-injection). In our study we used a less severe contusion injury but found a lower BMSC survival at time points that were close (acute injection – 28 days survival here vs. 35 days survival) or exactly matching (7 days delayed injection – 28 days survival here vs. 35 days survival), namely 0.02 vs. 0.17 and 0 vs. 0.99, respectively. A possible explanation for this discrepancy is that in Hofstetter et al. the BMSC were injected 2 mm rostral and caudal as well as into
the contusion lesion. It was not studied whether the surviving BMSC were in fact those injected near rather than in the contusion as has been described for a similar paradigm in a 7-day old mouse spinal cord contusion model. Others have reported survival of BMSC in spinal cord lesions but without quantification. Hofstetter et al. injected BMSC into the contused spinal cord acutely or one-week delayed and examined during the ensuing weeks locomotor function of the hind limbs. Both groups were not directly compared and tested for significance, but the data from this study indicated that improved BMSC survival was associated with improved locomotor function. In general, this agrees with our data that improved survival of transplanted BMSC elicits positive effects on spinal cord repair. Studies that investigate the correlation between BMSC survival and motor and sensory function are in progress.

Survival of grafted BMSC during the first week post-injection was significantly lower when the cells were grafted 7 and 21 days post-injury compared to 15 min and 3 days post-injury. Normally, immune cells invade contused tissue rapidly to be followed by inflammatory cells including macrophages. The peak in macrophage presence is around 7 days post-injury, to recede thereafter. We found that BMSC survived best when injected immediately or 3 days after injury, which is when the number of inflammatory cells is still increasing in the contusion lesion. Possibly, macrophages are directly involved in transplanted BMSC loss which would explain the greater loss at injection times when more macrophages can be found in the contusion. The relationship between macrophages and transplanted BMSC within the injured spinal cord is still poorly understood.

We observed that some of the transplanted BMSC migrated from the contusion into the rostral and caudal white matter. To our knowledge, this is the first evidence that BMSC transplanted into a contusion site in the adult rat thoracic spinal cord migrate into the adjacent nervous tissue. Neuhuber and colleagues described that human BMSC grafted in a hemisection in the adult rat cervical spinal cord migrated into the nearby spinal tissue. Our data suggested that the conditions at and nearby the 3-day old spinal cord contusion permitted migration of the injected BMSC. In the injured adult central nervous system, chemotactic factors and cytokines such as macrophage inflammatory protein-1, monocyte chemoattractant protein-1, and interleukin-8 have been implicated in BMSC migration.

In sum, we have gathered evidence that BMSC transplanted immediately and 3 days after a contusion survive better within the lesion environment during the first week after injection compared to BMSC transplanted at 7 and 21 days post-injury. The improved survival is transient but the BMSC transplant elicited neuroprotective effects that resulted in improved tissue sparing up to 28 days after injection.
Reducing macrophages to improve bone marrow stromal cell survival in the contused spinal cord.

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INTRODUCTION
A contusive spinal cord injury causes immediate death of neural cells and disruption of axon circuits\(^5\). The number of macrophages in the injury site rapidly increases over the ensuing days and contribute to further destruction of local nervous tissue\(^3\). There is no therapy available that effectively improves function after spinal cord injury.

Transplantation of mesenchymal bone marrow stromal cells (BMSC) has been explored for spinal cord repair\(^2\). In different model systems, BMSC transplantation resulted in tissue sparing and, in some cases, motor function\(^7\), \(^3\), \(^4\), \(^5\), \(^6\), \(^7\), \(^8\). These results warrant further investigation of BMSC for nervous system repair.

Survival of allogeneic BMSC transplanted into the damaged spinal cord is low\(^7\), \(^3\), \(^4\). Several studies have pointed at a role of macrophages in the loss of BMSC after injection into the injured nervous system\(^9\), \(^4\). So far, quantitative evidence that would support such a role is sparse.

Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment decreases macrophage infiltration into a spinal cord lesion\(^2\), \(^7\), \(^7\), \(^8\), \(^9\), \(^10\). The present study focuses on the efficacy of these three drugs to reduce macrophage infiltration and whether this would result in increased survival of subsequently transplanted BMSC.

MATERIAL AND METHODS

Spinal cord contusion and post-surgery care
Adult female Sprague-Dawley rats (n=76, 200-230g; Harlan, Indianapolis, IN, USA) were anaesthetized with an intraperitoneal injection of 60 mg/kg of Ketamine HCl (Phoenix Pharmaceuticals, St. Joseph, MD, USA) and 0.4 mg/kg meditomidine (Domitor, an alpha-2-adrenergic agonist; Orion Corporation, Espoo, Finland). The 10\(^{th}\) thoracic spinal cord segment was exposed and contused using the Infinite Horizon Impactor at a force of 200 kDyn (Fig. 1A). Consistency between animals was guaranteed by registering the impact force and spinal cord displacement. The wound was closed and the rats were given antisedan (atipamezole hydrochloride; 1.25 mg/kg, intramuscular), an alpha 2-adrenergic antagonist that reverses sedative and analgesic effects of meditomidine. All surgical procedures were performed by the same investigator. Post-surgery maintenance was as described previously\(^2\).
Inflammation and cell survival

Drug administration
Contused rats were divided into 4 groups that received CsA, MC, MP, or saline (n=18 each; Fig. 1A) starting 5 min after the contusion. All injections were performed by the same investigator. CsA (Bedford Labs, Bedford, OH, USA) was administered subcutaneously once per day at a dose of 30 mg/kg for the first three days and 15 mg/kg for the next seven days. MC (Sigma-Aldrich, St. Louis, MO, USA) was administered intraperitoneally at a dose of 50 mg/kg twice a day for the first two days. MP (Sigma-Aldrich) was administered intraperitoneally once at a dose 30 mg/kg. Saline was given to controls following the same regime as for MP.

BMSC culture and lentiviral transduction
BMSC were obtained from femurs of adult female Sprague-Dawley rats (n=4) as previously described (Fig. 1B). BMSC at passage 0 were transduced overnight using lentiviral vectors encoding for green fluorescent protein (GFP) at an MOI of 150. Transduced BMSC were cultured in D-10 medium at 37 °C/5 % CO₂. BMSC from the third passage (Fig. 1C) were used for the transplantation experiments. The transduction rate of the BMSC was determined using a FACScan/FACSorter (Becton Dickinson Immunocytometry Systems (BDIS) Biosciences, San Jose, CA). The number of viable (GFP-positive) cells relative to the total number of cells was determined revealing a transduction rate of 63 %.

Fig. 1. Schematic representation of the experiments and BMSC harvest and transduction. (a) Rats were contused at the 10th thoracic spinal cord segment and then divided into 4 groups that received CsA, MC, MP or saline. (b) BMSC were harvested, transduced to express GFP using lentiviral vectors, and grown in DMEM. (c) GFP-expressing BMSC in a passage 3 culture which were used for transplantation into the epicenter of the 3-day old contusion. Abbreviations: BMSC, bone marrow stromal cells; CsA, cyclosporine A; ip, intraperitoneal; MC, minocycline; MP, methylprednisolone; sc, subcutaneous. Bar in C represents 10 μm.
Chapter 5

Transplantation of BMSC

At three days after injury, 24 rats (6 rats from each group) were anaesthetized with intraperitoneal injections of 60 mg/kg of Ketamine HCl (Phoenix Pharmaceuticals) and 0.4 mg/kg of meditomidine (Domitor; Orion Corporation). The 10th thoracic spinal cord segment was exposed and 5 μl DMEM with 1 x 10⁶ BMSC (Fig. 1A) was injected into the contusion epicenter. Four extra contused rats were similarly injected and perfused with fixative (see below) 15 min later. These rats were used to determine the number of BMSC in the contusion at 15 min post-injection. All BMSC injections were performed by the same investigator. After the injections, the rats were maintained as described previously.

General histology

Three days (n=24) and ten days (n=48) after injury, rats were anaesthetized with an intraperitoneal injection of 90 mg/kg of Ketamine HCl (Phoenix Pharmaceuticals) and 0.6 mg/kg of meditomidine (Orion Corporation). After deep sedation was confirmed, 0.1 ml Heparine (500 IU; Henry Schein, Melville, NY, USA) was injected into the left ventricle of the heart. Then, 500 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) was pumped through the vascular system. Spinal cords were removed without damaging the anatomical integrity, post-fixed for 24 h in the same fixative, and transferred to 30% sucrose in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) for 48 h. A 12 mm long spinal cord segment centered at the contusion was cut into 20 μm thick horizontal cryostat sections which were mounted on glass slides.

Immunocytochemical procedures

For characterization of the BMSC, 8-well chamber glass slides (BD Falcon; BD Biosciences, Bedford, MA) were coated with 100 μl/ml poly-D-lysine for 1 h at room temperature. After washing 2 x 5 min with double-distilled water, 3000 BMSC in 250 μl D-10 medium were plated per well. After two days at 37 °C/5 % CO₂, the cultures were washed 3 x 5 min with PBS and fixed with 4% paraformaldehyde in PB (10 min, room temperature). Next, the cultures were washed 5 x 5 min with PBS, incubated with 5% normal goat serum (NGS) in PBS for 30 min, and then incubated overnight at 4 °C with antibodies against CD90 (1:100; Immunotech, Brussels, Belgium), CD105 (1:100; Ni, C., Becton Dickenson), CD34 (1:100; 8G12 clone IgG₁, Becton Dickenson), CD45 (1:100; H130 clone IgG₁, Becton Dickenson), and HLA-DR (1:100; Dako, Glostrup, Denmark) diluted in PB with 5% NGS. Some cultures were incubated with PBS with 5% NGS only and served as negative (no primary antibody) controls to exclude a false-positive outcome. Next, cultures were washed 3 x 5 min with PB and then incubated with goat-anti-mouse IgG-Alexa 594 (1:500 in PB; Molecular Probes, Carlsbad, CA) for 2 h at room temperature. Afterwards, cultures were washed 3 x 5 min with PB and covered with a glass slip.
with Vectashield and DAPI (Vector Laboratories, Inc., Burlingame, CA). The slides were examined and images were taken with an Olympus Fluoview FV1000 confocal microscope.

For immunostaining of activated macrophages, every 10th cryostat section was pre-incubated at room temperature for 30 min in 5% NGS and 0.3% Triton X-100 in 0.01 M PBS (pH 7.4) and then incubated with antibodies against ED1 (1:200; Serotec, Raleigh, NC) in 5% NGS for 2 h at room temperature followed by overnight incubation at 4 °C. After washing 3 x 5 min with PBS, sections were incubated with goat anti-mouse Alexa 594 antibodies (1:200; Molecular Probes) in PBS (0.01 M; pH 7.4) at room temperature for 2 h. The sections were then washed and covered with a glass slip in Vectashield with DAPI (Vector Laboratories, Inc.). The coverslips were sealed with nail polish. All sections were stored at -20 °C until analysis.

Quantitative assessments
For analysis of GFP-positive BMSC, every 10th cryostat section was covered with a glass slip with Vectashield mounting medium with DAPI (Vector Laboratories, Inc). Stereoinvestigator (MicroBrightField Inc., Colchester, VA, USA) was used to determine the numbers of surviving BMSC in the contusion. The sections were 200 mm apart spanning the width of the spinal cord. In every section containing GFP positive cells, the transplanted area was outlined manually at 4X magnification and covered by a 250 x 250 μm grid. At 60X magnification with oil immersion, GFP-positive cells with a discernable DAPI-positive nucleus were marked using the optical fractionator with a 60 x 60 μm counting frame. Numbers were corrected for the 63% transduction rate of the BMSC with LV-GFP. For each of the groups, BMSC survival was calculated as the number of BMSC relative to the number of BMSC at 15 min after transplantation (which was 168159 ± 31129; SEM, n=4). The effect of treatment on BMSC survival was assessed by expressing BMSC survival for each group as a percentage of that in controls. For analysis of macrophages we employed a method previously employed Hayashi and colleagues. This method uses three sections per rat for examination: one section through the center of the contusion/transplant (with the densest cellular staining), and sections 200 μm dorsal and ventral to the center. The area fraction of staining in these sections was determined using SlideBook 4.1.0.12 (Intelligent Imaging Innovations, Inc, Santa Monica, CA, USA) and expressed as a percentage of that in control animals.

Statistical analysis
Sigmastat® (Systat Software, Inc., San Jose, CA, USA) was used for statistical analyses using one-way ANOVA and the Bonferroni post-hoc test. Differences were accepted at p<0.05.
**Ethics and surgical approval**

All rats used in this study were housed according to the guidelines of the National Institute of Health and United States Department of Agriculture. The described animal procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University.

**RESULTS**

**Characterization of BMSC in vitro**

Cultured GFP-positive cells expressed CD90 (Fig. 2A) and CD105 (Fig. 2B). These two extracellular molecules are both well-known BMSC markers. None of the cells expressed the blood cell markers, CD34 (Fig. 2C) and CD45 or the immune cell marker, HLA-DR. No staining was visible if the primary antibody was omitted. The data characterize the cells used for transplantation as BMSC.

![Figure 2](image.png)

**Fig. 2. Characterization of BMSC in vitro.** Cultured cells expressed the BMSC markers, CD90 (a) and CD105 (b), but not the blood cell marker, CD34 (c). Scale bar=10 μm.

**CsA, MC, and MP reduces macrophage infiltration into the spinal cord contusion**

Microscopic analysis of macrophage presence in the contusion revealed high numbers in controls (Fig. 3a) compared to treated rats (Fig. 3b) at 3 days after injury. The numbers appeared increased in treated rats at 10 days (Fig. 3c) compared to 3 days (Fig. 3b) after contusion. Quantitative analysis demonstrated that at three days post-injury, relative to controls, macrophage infiltration was 46 ± 10 % with CsA-, 47 ± 3 % with MC-, and 63 ± 3 % with MP-treatment (Fig. 3d). ANOVA revealed that the number for each of the treatment groups was significantly smaller (p<0.001) than that for controls. Thus treatment-induced reduction was 54 % with CsA, 53 % with MC, and 37 % with MP compared to controls.
Treatment-induced reduction was not significantly different from controls at ten days post-injury (Fig. 3d). These results showed that all three drugs when administered following the treatment regime described above reduced macrophage infiltration into the adult rat spinal cord contusion at three days post-injury.

**BMSC survival in contusion is not affected by CsA, MC, or MP treatment**

BMSC were present in the contusion at seven days post-injection (Fig. 4a). Quantitative analysis revealed that BMSC survival in the contusion at seven days post-injection was $27 \pm 4\%$ with CsA, $24 \pm 4\%$ with MC, and $33 \pm 2\%$ with MP treated rats. In saline-injected control rats, BMSC survival was $21 \pm 7\%$. To assess the effects of treatment, we expressed BMSC survival in each treatment group relative to that in the control group (Fig. 4b). We found that BMSC survival was $126 \pm 17\%$ with CsA, $111 \pm 19\%$ with MC, and $155 \pm 12\%$ with MP treated rats relative to control rats (Fig. 4b). ANOVA revealed no difference in BMSC survival between treatment and control groups ($p=0.16$).

![Fig. 3. Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment reduced macrophage infiltration into the contusion. Photomicrographs of ED-1-positive cells in control rats (a) and CsA-treated rats (b) at 3 days post-contusion and in CsA-treated rats at 10 days post-contusion (b). In panel (c) the more intense staining was found associated with cellular debris. (d) Bar graph shows that at 3 days post-injury (open bars) relative to controls (Con), macrophages infiltration in the contusion was decreased significantly with CsA, MC, and MP treatment. At 10 days post-injury (solid bars), the decrease in macrophage presence in the contusion of treated rats was not statistically different from that in Con. *Significant difference between treated and Con groups at 3 days post-injury with $P<0.001$.](image-url)
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Fig. 4. Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment did not improve bone marrow stromal cell (BMSC) survival. (a) Photomicrograph of green fluorescent protein-positive cells within the contusion at 7 days postinjection. (b) Bar graph showing BMSC survival relative to controls (Con). The differences were not statistically different, although there was a trend towards higher numbers in CsA-treated rats.

**BMSC presence initiates macrophage infiltration into the spinal cord contusion.** We assessed the effect of a BMSC transplant on macrophage presence in the contusion at ten days post-injury relative to that at three days post-injury. With a BMSC transplant, macrophage infiltration relative to controls was 2.4 fold in CsA- and MP-treated rats, and 6.9 fold in MC-treated rats. These differences were statistically significant (p<0.001). In the absence of a BMSC transplant, macrophage presence in treated rats relative to controls was unchanged. Our data demonstrated that the presence of a BMSC transplant significantly increased macrophage infiltration into the contused adult rat spinal cord.

**DISCUSSION**

Quantitative investigations have demonstrated that survival of BMSC transplanted into the contused adult rat spinal cord is low. Previously, it was proposed that macrophages which are naturally present within an injury site are involved in the loss of BMSC transplanted into the central nervous system. Our present results showed that a decreased presence of activated macrophages at the time of BMSC injection (three days post-injury) does not increase survival of grafted BMSC. It is possible that our treatment regimens failed to lower macrophage infiltration to a level where BMSC survival would have been improved. The maximum reduction which was achieved here was 54%. Because macrophage invasion into a spinal cord injury site is typically large, this reduction may still leave many macrophages that could potentially contribute to BMSC loss. An additional observation is that the treatment effect which was present at three days post-injury was not significant at ten days post-injury.
Inflammation and cell survival

Although we used treatment protocols known to effectively reduce the presence of macrophages they might not have been effective enough for sufficient and prolonged reduction of macrophages.

An alternative explanation for the observed lack of improved BMSC survival would be that any treatment-induced reduction in macrophage presence was masked by a subsequent increase in macrophage infiltration due to the introduction of BMSC into the environment. This notion is supported by our data because in animals with a BMSC transplant we found that macrophage infiltration was drastically increased. It is likely that these extra macrophages have exacerbated the loss of BMSC. It is important to keep in mind that other factors than invaded macrophages are most likely also involved in transplanted BMSC loss such as the lack of oxygen and/or nutrients within the damaged tissue.

Previously, it was reported that BMSC are hypo-immunogenic; they suppress the proliferation and function of T-cells, B-cells, natural killer cells, and dendritic cells. However, these publications did not investigate possible effects of BMSC on macrophage invasion. It is possible that the immunosuppressive properties of BMSC affect only the adaptive immunity due to the low expression level of human leukocyte antigen (HLA) major histocompatibility (MHC) class I and the absence of co-stimulatory molecules. This would explain why the recruitment of macrophages (acquired immunity) would not be affected by BMSC.

Lowering the number of macrophages in the injured spinal cord needs to be addressed with caution. It is well known that macrophages can support spinal cord repair by promoting axon regeneration and myelination which may be accompanied by improved function. These constructive effects occur while macrophages also exert destructive effects such as neural cell death. Because of this dual role, decreasing the number of macrophages within a spinal cord injury could lead simultaneously to beneficial and detrimental effects. Thus it is important to aim for a reduction in macrophages that would not jeopardize their positive contributions to spinal cord repair.

CONCLUSION

We hypothesized that a decreased macrophage presence in an adult rat spinal cord contusion would support survival of a BMSC transplant. With CsA, MC, and MP treatment we successfully lowered macrophage infiltration; however, this reduction, which was maximum 54 %, did not improve transplanted BMSC survival. Surprisingly, we found that BMSC presence in the contusion in reality increased macrophage presence by almost 4-fold. Based on our current knowledge it is to be expected that macrophages are involved in the loss of BMSC after injection into the injured spinal cord. The finding that a large decrease in macrophage
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infiltration failed to improve survival of subsequently injected BMSC, point at key roles of other injury-related events in the loss of an intraspinal BMSC transplant.
Functional recovery after transplantation

Locomotor and sensory function recovery after autologous bone marrow stromal cell transplantation in the contused adult rat spinal cord.

Submitted Cell Transplantation

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Chapter 6

INTRODUCTION
Contusive injury to the adult rat thoracic spinal cord causes immediate locomotor and sensory impairments of the hindlimbs\(^{23, 146, 408}\). Locomotor function gradually improves until a plateau is reached after 2-5 weeks depending on the impact severity\(^{24, 60, 202, 330}\). Spontaneous restoration of sensory function is mostly absent\(^{176, 419}\) but some recovery was reported to occur months after the impact\(^{37}\).

Transplantation of repair-promoting cells into the contused spinal cord has been extensively explored as an intervention to restore function over what is spontaneously observed\(^{29, 122, 313, 329, 338}\). One of the candidate cell types for spinal cord repair is the bone marrow stromal cell (BMSC). These cells are relatively easy to obtain via a bone marrow biopsy which warrants their given promise for clinical application\(^{285, 375, 430}\).

Currently, the ability of BMSC transplants to restore function after spinal cord contusion is debated. Some investigators reported beneficial effects on motor function\(^{169, 179, 477, 473}\) but others are in disagreement\(^{195, 384, 461}\). Spinal cord tissue sparing has been proposed as a probable mechanism of BMSC-mediated repair\(^{386, 394}\). Effects of a BMSC graft on sensory function restoration after spinal cord contusion has not been described.

MATERIAL AND METHODS

Ethics and surgical approval
All rats used in this study were housed pre- and post-surgery according to the National Institutes of Health and the United States Department of Agriculture guidelines. Air in the cages was continuously refreshed and water and food were available ad libitum. At all times during the experiment, rats were kept within a double-barrier facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University.

BMSC culture and lentiviral transduction
BMSC were harvested from the marrow of the femurs of adult female Sprague-Dawley rats (n=4, 200-230g; Harlan, Indianapolis, IN, USA) according to previously described protocols\(^{15, 359}\). Lentiviral vectors encoding for green fluorescent protein (GFP) were prepared as previously described\(^{275}\) and used at an MOI of 150 to transfec BMSC at passage 0. For transplantation, BMSC from passage 4 were used of which 63% expressed GFP as determined by FACScan/FACSorter (Becton Dickinson Immunocytometry Systems Biosciences, San Jose, CA, USA). Previously, we showed that over 95% of the cells we used for intraspinal injection expressed CD90 and CD105, which are typical BMSC markers, and none of them expressed CD34 and CD45, both blood cell markers, or HLA-DR, an immune cell marker\(^{359}\).
**Pre-surgery procedures**

Adult female Sprague-Dawley rats ($n=40$, 200-230g; Harlan) were sedated with intraperitoneal injections of 60 mg/kg Ketamine HCl (Phoenix Pharmaceuticals, St. Joseph, MD, USA) and 0.4 mg/kg medetomidine (Domitor®, an alpha-2-adrenergic agonist; Orion Corporation, Espoo, Finland). After deep sedation was verified, the back of the rats was shaved and cleaned with Betadine and 70% alcohol. Lacrilube ointment was applied to the eyes, and 6 mg/kg gentamicin (Abbott Laboratories, North Chicago, IL, USA) was administered intramuscularly.

**Spinal cord contusion**

The lower thoracic (T) spinal column was exposed, the lamina of the T9 was removed, and the exposed underlying T10 spinal cord segment contused using the Infinite Horizon Impactor at a force of 200 kDyne. Consistency between rats was guaranteed using the records on the compression rate and velocity of the impactor; rats from which these values deviated over 5% were excluded from the study. The wound site was rinsed with phosphate-buffered saline (PBS) with 0.1% gentamicin (Abbott Laboratories) and the muscles were closed in layers using 5.0 sutures. The skin was closed with Michel wound clips.

**Post-contusion procedures**

After closure of the wound, the rats received a subcutaneous injection of 1.5 mg/kg atipamezole hydrochloride (antisedan®; an alpha 2-adrenergic antagonist; Pfizer Inc., New York, NY, USA), to reverse the sedative and analgesic effects of medetomidine. Ten ml lactated Ringer’s solution was injected subcutaneously and 6 mg/kg gentamicin (Abbott Laboratories) intramuscularly. The rats were kept in a small animal incubator at 37°C until full recovery and were then returned to their cages. Until the second surgery at three days post-contusion, the rats received daily 5 ml Ringer’s solution (subcutaneous), 6 mg/kg gentamicin (Abbott Laboratories; intramuscularly), and 0.03 mg/kg Buprenorphin (Buprenex®; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA; subcutaneously). The bladder was manually emptied twice per day.

**CsA administration**

Starting 5 min after the contusive injury, contused rats received CsA (Bedford Labs, Bedford, OH, USA) subcutaneously once per day at a dose of 30 mg/kg for the first three days after contusion injury and 15 mg/kg for the following days throughout the survival period. Other contused rats received saline injections.
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**BMSC transplantation**

At three days after contusion, rats were sedated with intraperitoneal injections of 60 mg/kg Ketamine HCl (Phoenix Pharmaceuticals) and 0.4 mg/kg meditomidine (Orion Corporation). The T10 spinal cord segment was re-exposed and $1 \times 10^6$ BMSC in DMEM or DMEM alone (total volume was 5 μl in both cases) was injected into the contusion epicenter using a Hamilton syringe with a pulled glass needle attached (tip diameter: 150 μm) fixed within a micromanipulator. After injection, the wound was closed as described above.

**Post-injection procedures**

After closure of the wound, rats were fully recovered in a small animal incubator at 37°C before being returned to their cages. The rats received 5 ml Lactated Ringer’s solution daily for 2 days (subcutaneously) and 6 mg/kg gentamicin (Abbott Laboratories) daily for seven days post-injection (intramuscularly). The rats were injected with 0.03 mg/kg Buprenorphin (Reckitt Benckiser Pharmaceuticals Inc.; subcutaneously) daily for the first 3 days post-injection. Bladders were manually emptied twice per day until reflex voiding started. Throughout the remainder of the experiment the rats were monitored daily. In case of pain or distress rats were given 0.03 mg/kg Buprenorphin (Reckitt Benckiser Pharmaceuticals Inc.) subcutaneously daily for 3 days.

**Experimental groups**

Rats with a T10 contusion received either BMSC/DMEM or DMEM only and were treated with CsA or saline. Thus there were 4 experimental groups: BMSC/CsA (n=9), BMSC/saline (n=8), DMEM/CsA (n=12), and DMEM/saline (n=10).

**Testing of locomotor function**

All rats were included in locomotor function testing. Automated hindlimb movements were analyzed for eight weeks post-contusion using the Basso-Beattie-Bresnahan (BBB) test. Rats were familiarized with the open field before injury. Three days after the contusion, just before injection, rats were tested and those with a score above 5 were removed from the study. Rats were tested once a week for eight weeks after the injection into the contusion epicenter over a period of 4 min by two examiners oblivious of the treatments.

Specific locomotion-related features were assessed using the BBB sub-score. Paw position (parallel initially or at liftoff) and toe clearance (occasional, frequent, or consistent) for each hind paw, and the tail position (up, down) and trunk stability (yes, no) were determined. The scores of both limbs and tail and trunk was summed (maximum score possible was 8).

The pattern of locomotion was assessed using the footprint analysis which was modified from that of De Medinaceli and colleagues. Hindpaws were inked and footprints were made
on paper within a runway of about 1 m length and 7 cm width. The prints were used to measure stride length, base of support, and angle of paw rotation. Average values per paw were calculated from at least 5 sequential steps. Values for both paws were averaged. Stride length was defined by the distance between the central pads of two consecutive prints on each side. Base of support was determined by measuring the core to core distance of the hind paws central pads. Limb rotation was defined by the angle formed by the intersection of the line through the print of the third digit and the print representing the metatarsophalangeal joint and the line through the central pad parallel to the walking direction. Footprint testing was performed at 4 and 8 weeks after transplantation at which times all rats exhibited weight support.

Sensorimotor function of the hindlimbs was assessed at four and eight weeks post-contusion using the horizontal ladder test218. We used a 100 cm long horizontal ladder which the rats crossed three times each test. The passages were videotaped and later played back for accurate evaluation. Only the middle 60 cm of the ladder was used for measurements. Small (foot or part of foot), medium (foot and part of lower leg), and large (full leg) slips were counted and expressed as a percentage of the total number of steps.

**Testing of sensory function**
Mechanical allodynia was determined by measuring foot withdrawal in response to a normally innocuous mechanical stimulus applied with an (electronic) von Frey aesthesiometer69, 70. During the test, rats were in a plexiglas box with an elevated mesh floor. The rats were acclimated for 5 min before testing. The von Frey tip was applied perpendicularly to the mid-plantar area of each hind paw and depressed until paw withdrawal, at which time the pressure (in g) was recorded (3 times each test). The values for both paws were averaged.

Withdrawal response to a normally innocuous heat source, applied using a Hargreave’s heat source, was used to test thermal hyperalgesia165. The rats were kept in a plexiglas box with an elevated mesh floor for 5 min to acclimate. The radiant heat source with constant intensity was aimed at the mid-plantar area of each hind paw. The time (in sec) from initial heat source activation to paw withdrawal was recorded. A second and third measurement was performed 5 and 10 min later. The values from both paws were averaged.

**Statistical analysis**
One-way analysis of variance (ANOVA) followed by Fisher’s protected least-significant difference (PLSD) test was used to determine statistical differences between groups. In case of unequal variance (F test), a nonparametric analysis (Kruskal-Wallis test followed by Mann-Whitney U-test) was used. Statistically significant differences were accepted at P<0.05.
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RESULTS

Surgery data
Forty-eight rats were included in this study. All rats were contused and three days later injected with BMSC/DMEM (n=17) or DMEM only (n=22) into the contusion epicenter. Two rats died prior to the transplantation surgery. Seven rats were removed from the study because their BBB score was higher than 5 at 3 days post-contusion (n=5) or their impact parameters were over 5% off from the intended values (n=2). None of the injected rats died during their survival period.

BMSC transplants did not improve open field locomotor ability
Automated open field locomotor function was evaluated using the BBB test[^23] and analyzed using two-way ANOVA, which did not show any differences between treatment groups and the control group. The average BBB score of control (DMEM/saline) rats was 11.0 ± 0.0 (SEM) at eight weeks after injection into the contusion. The other groups had similar scores; 11.4 ± 0.2 for the BMSC/saline group, 11.1 ± 0.1 for the DMEM/CsA group, and 10.9 ± 0.1 for the BMSC/CsA group (Fig. 1A). All rats reached the plateau score of 11 at 3-4 weeks post-impact, which was consistent with previously published data for rats with a similar contusion only[^60]. The score of 11 reflects the ability to support their weight on their hindlimbs and to make frequently to consistently plantar steps without forelimb-hindlimb coordination.

BMSC transplants increased open field locomotion-related features (BBB sub-score).
The sensitivity of the BBB scale can be increased by evaluating individual characteristics of locomotor function using the BBB sub-scoring scale[^23]. Two-way ANOVA showed significant differences across treatment groups (F (3,252) = 55.6; p<0.0001). For control rats the BBB sub-score was 1.4 ± 0.3 (SEM) at eight weeks after injection. Rats with a BMSC transplant exhibited a score of 5.7 ± 0.3 which reflected a significant 4-fold increase compared to controls. The difference between these BMSC treated rats and controls became significantly noticeable starting three weeks post-transplantation (Fig 1B). Between three and six weeks after transplantation, CsA treated rats had higher sub-scores than controls but this effect was no longer present at seven or at eight weeks. The scores in rats with BMSC and CsA were not different from controls.
Functional recovery after transplantation

**BMSC transplants improved some aspects of the pattern of locomotion**

Footprint analysis was used to investigate changes in stride length, base of support, and angle of paw rotation which are features of the pattern of locomotion. At eight weeks after injection into the contusion, one-way ANOVA showed no differences among groups in stride length \( (F \ (3.14) = 1.17; p=0.35; \text{Fig. 2A}) \) or in base of support \( (F \ (3.17) = 1.94; p = 0.16, \text{Fig. 2B}) \). One-way ANOVA did reveal differences in angle of hind paw rotation \( (F \ (3.15) = 8.29; p=0.002, \text{Fig. 2C}) \). Tukey post-hoc comparison showed that a BMSC transplant results in a 26% smaller angle of rotation \( (M = 19.7; 95\% \ CI [18.7, 20.7]) \) than control animals \( (M = 26.5; 95\% \ CI [26.0, 27.0]; \text{Fig. 2C}) \). Rats that received CsA had a 19% smaller angle of rotation \( (M = 21.5; 95\% \ CI [20.5, 22.5]; \text{Fig. 2C}) \) compared to controls and rats that received the combination of BMSC and CsA showed a 36% smaller angle of rotation \( (M = 16.9; 95\% \ CI [15.9, 17.9]; \text{Fig. 2C}) \).
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BMSC transplants improved sensorimotor function. After a contusion injury to the spinal cord, rats exhibit impaired integration of sensory and motor inputs. Changes in sensorimotor function were tested with a horizontal ladder by counting the number of slips off the rungs by the rat’s hind paw (small slip), hind paw and part of leg (medium slip) or whole hind leg (large slip). The number of slips were quantified and expressed as a percentage of the total number of steps. The average number of steps was $15.0 \pm 0.3$ at eight weeks. One-way ANOVA showed significances across groups ($F(3,23) = 42.7; p < 0.0001$). Control rats made slips in $70.3 \pm 4.7$% of their steps at eight weeks (Fig. 3), which was significantly different from BMSC treated animals, who showed a $70$%
improvement ($M = 21.4; CI [18.8, 24.0]$; Fig. 3). Rats with BMSC and CsA treatment showed a 52% improvement from controls ($M = 33.8; CI [31.2, 36.4]$) and rats that received CsA treatment only showed a 26.6% improvement from controls ($M = 52.3; CI [49.6, 55.0]$).

**Fig. 3.** Presence of a BMSC transplant improved performance on the horizontal ladder. The number of small, medium, and large slips was assessed individually. These numbers were also added and expressed as a percentage of the total number of steps necessary to cross the horizontal ladder. The assessment was done at 8 weeks post-transplantation. Rats with a BMSC transplant (white bar) had a significant better performance (i.e., lower percentage of slips/steps) than control rats (black bars) (asterisks, $p<0.05$). This was also the case for rats with CsA treatment (dark gray bars) and rats with BMSC and CsA (light gray bars). The error bars indicate the standard deviation of the mean.

**BMSC transplants improved recovery from mechanical allodynia.**

After a thoracic contusive spinal cord injury rats develop allodynia of the hind paws. An (electronic) von Frey anesthesiometer was used to test whether a BMSC transplant would be beneficial for recovery from mechanical (tactile) allodynia. One-way ANOVA ($F(3,23) = 24.4; p<0.0001$) and Tukey’s post-hoc comparisons showed that at eight weeks post-transplantation, an improvement of 21% in recovery from mechanical allodynia was observed in rats with a BMSC transplants ($M = 88.0; CI [85.8, 90.2]$), compared to controls ($M = 58.2; CI [56.5, 59.9]$; Fig. 4A). Treatment with CsA ($M = 72.8; CI [70.2, 75.4]$) or BMSC and CsA combined ($M = 70.7; CI [69.6, 71.8]$) did not change the withdrawal response compared to controls (Fig. 4A).
Fig. 4. Presence of a BMSC transplant decreases mechanical and thermal allosthenia. Allosthenia of the hind paws was assessed using a von Frey anaesthesiometer (A) and a Hargreave’s heat source (B) at 8 weeks post-transplantation. A. Rats with a BMSC transplant but none of the other treatment groups had significantly higher response times (i.e., lowered (improved) hyper-sensitivity) to a mechanical (tactile) hind paw stimulus compared to the control rats (asterisks, p<0.05). B. The response times of rats with a BMSC transplant was significantly higher than in controls (asterisks, p<0.05), which indicated lowered (improved) hyper-sensitivity to a thermal hind paw stimulus. In both graphs the error bars indicate the standard deviation of the mean.

Fig. 5. The presence of a BMSC transplant improved the amount of spared tissue. The volume of spared tissue was assessed at 8 weeks post-transplantation and expressed as a percentage of the volume of an uninjured comparable spinal cord segment. We found that rats with a BMSC transplant had significantly more spared tissue than control rats (asterisks, p<0.05). The other treatment groups were not different from the control group. The error bars indicate the standard deviation of the mean.
**BMSC transplants improved recovery from thermal allodynia**

Contused rats also develop thermal allodynia, which can be measured with a Hargreave’s heat source\(^6\). One-way ANOVA showed differences across groups (\(F(3, 23) = 20.7; p < 0.0001\)). Rats with a contusion only (no treatment) withdrew their hind paws at 6.1 ± 0.3 s (Fig. 4B). Rats that received the BMSC transplant withdrew their hind paw at 7.6 ± 0.3 s which represented a 25% improvement over controls (Fig 4B). The other groups were not different from the control group (Fig 4B).

**BMSC transplants elicited tissue sparing.**

Spared tissue volumes at the contusion site were measured at eight weeks after injection into the contusion using Stereo Investigator\(^\text{®}\) software (MBF Bioscience) in a blinded fashion. For all measurements the Coefficient of Error (Gundersen Coefficient) was ≤0.05. The volumes were expressed as a percentage of the volume of a comparable segment of a naive (uninjured) spinal cord. One-way ANOVA showed differences across groups (\(F(3, 19) = 20.7; p = 0.0005\)) In control rats (contusion only) the volume of spared tissue was 14.9 ± 1.6 % (SEM) of that of an uninjured spinal cord (Fig. 5). With a BMSC transplant the volume was 24.8 ± 1.4 %, which represented a significant 66 % increase in spared tissue volume compared to controls (\(p < 0.05\); Fig. 5). In rats with daily CsA treatment the spared tissue volume was 17.8 ± 1.7 % and in rats with a BMSC transplant and CsA treatment the volume was 19.2 ± 1.6 % of that of an uninjured spinal cord segment (Fig. 5). The volumes in these two groups were similar as the volume in control rats.

**Correlations between tissue sparing and motor and sensory function.**

The relationship between tissue sparing and motor and sensory function per treatment group (Table 1) and per function (Table 2) was assessed using the Pearson correlation coefficient (\(r\)). BMSC transplantation resulted in a strong association (\(r > 0.75\)) between tissue sparing and BBB sub-score, horizontal ladder walking, angle of rotation, and mechanical allodynia. A moderate association (\(r > 0.50, < 0.75\)) was found between tissue sparing and thermal allodynia (Table 1). With CsA treatment tissue sparing was strongly associated with the angle of rotation, moderately with BBB sub-score, and weakly (\(r < 0.50\)) with horizontal ladder walking, mechanical allodynia, and thermal allodynia (Table 1). With both BMSC transplantation and CsA treatment tissue sparing was strongly associated with the angle of rotation, moderately with the BBB sub-score, horizontal ladder walking, and thermal allodynia, and weakly with mechanical allodynia (Table 1). In our control rats the relationship was strong between tissue sparing and thermal allodynia but weak for all other functions.

If all four treatment groups were pooled together per specific function (Table 2), tissue sparing was strongly associated with BBB sub-score (Fig. 6A), moderately associated with
horizontal ladder walking (Fig. 6B), angle of rotation (Fig. 6C), and thermal alldynia (Fig. 6D), and weakly associated with mechanical alldynia (Fig. 6E).

![Scattergrams showing tissue sparing](image)

Fig. 6. The amount of tissue sparing correlated with motor and sensory outcomes. The relationship between tissue sparing and motor and sensory function (with all four treatment groups pooled) was assessed using the Pearson correlation coefficient (r). Correlations between tissue sparing and individual groups are described in text. Scattergrams show tissue sparing was strongly associated with (A) BBB sub-score, moderately associated with (B) horizontal ladder walking, (C) angle of rotation and (D) thermal alldynia, and weakly associated with (E) mechanical alldynia.
### Table 1. Correlation between tissue sparing motor and sensory function. The relationship between the different outcomes per treatment group was determined by the Pearson correlation coefficient (r).

<table>
<thead>
<tr>
<th>Function</th>
<th>Treatment</th>
<th>r</th>
<th>Function</th>
<th>Treatment</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB</td>
<td>BMSC / saline</td>
<td>0.01</td>
<td>Foot Print (angle)</td>
<td>BMSC / saline</td>
<td>-0.92</td>
</tr>
<tr>
<td></td>
<td>DMEM / CsA</td>
<td>0.35</td>
<td></td>
<td>DMEM / CsA</td>
<td>-0.96</td>
</tr>
<tr>
<td></td>
<td>BMSC / CsA</td>
<td>-</td>
<td></td>
<td>BMSC / CsA</td>
<td>-0.95</td>
</tr>
<tr>
<td></td>
<td>DMEM / saline</td>
<td>-</td>
<td></td>
<td>DMEM / saline</td>
<td>-</td>
</tr>
<tr>
<td>BBB Subscore</td>
<td>BMSC / saline</td>
<td>0.86</td>
<td>Mechanical Allodynia</td>
<td>BMSC / saline</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>DMEM / CsA</td>
<td>0.73</td>
<td></td>
<td>DMEM / CsA</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>BMSC / CsA</td>
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<td></td>
<td>BMSC / CsA</td>
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</tr>
<tr>
<td></td>
<td>DMEM / saline</td>
<td>-</td>
<td></td>
<td>DMEM / saline</td>
<td>0.10</td>
</tr>
<tr>
<td>Horizontal Ladder slips/step</td>
<td>BMSC / saline</td>
<td>-0.85</td>
<td>Thermal Allodynia</td>
<td>BMSC / saline</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>DMEM / CsA</td>
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<td>DMEM / CsA</td>
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<td>BMSC / CsA</td>
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<tr>
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<td>DMEM / saline</td>
<td>-0.40</td>
<td></td>
<td>DMEM / saline</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Abbreviations: *r = Pearson correlation coefficient; BBB = Basso-Beattie-Bresnahan open field locomotor scale; BMSC = bone marrow stromal cells; CsA = cyclosporine; DMEM = Dulbecco’s minimal essential medium.

### Table 2. Correlation between tissue sparing motor and sensory function.

The relationship between the different outcomes was determined per specific function by the Pearson correlation coefficient (r). Abbreviations: *r = Pearson correlation coefficient; BBB = Basso-Beattie-Bresnahan locomotor scale.

<table>
<thead>
<tr>
<th>Function</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB</td>
<td>-</td>
</tr>
<tr>
<td>BBB Subscore</td>
<td>0.85</td>
</tr>
<tr>
<td>Horizontal Ladder</td>
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<td>Foot Print (angle)</td>
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<tr>
<td>Mechanical Allodynia</td>
<td>0.39</td>
</tr>
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<td>Thermal Allodynia</td>
<td>0.57</td>
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</tbody>
</table>
DISCUSSION

We evaluated locomotor and sensory recovery in rats with a contused thoracic spinal cord that received a BMSC transplant into the epicenter of the injury at three days post-impact. The main finding in our study is that the presence of a BMSC transplant results in improved motor and sensory function. Rats that received a BMSC transplant had improved BBB sub-scores, smaller angle of hind paw rotation, improved performance on the horizontal ladder, and less hypersensitivity to mechanical and thermal stimuli. BMSC-transplanted rats did not benefit functionally from daily CsA treatment. The amount of spared tissue at the contusion site in BMSC-transplanted rats, but not in any of the other groups, was increased significantly compared to controls. Moreover, rats with a BMSC transplant showed a strong correlation between the amount of tissue sparing and the functional outcome. Our data suggest that BMSC-mediated tissue sparing in the contused adult rat spinal cord is intimately involved in recover of motor and sensory function.

So far, the benefits of BMSC for motor function restoration had been unclear as several studies were at variance with earlier reports that described motor function improvements. We now demonstrate that BMSC transplantation has widespread benefits for motor function restoration after spinal cord contusive injury. A possible mechanism that may underlie these motor function improvements is BMSC-elicited nervous tissue sparing. Indeed we found that rats with BMSC had increased amounts of spared tissue at the contusion site and, interestingly, this strongly correlated with functional outcomes.

We did not observe a beneficial effect of transplanted BMSC on automated hind limb movements as assessed in the open field (BBB-test). Also, BMSC transplants had no effect on stride length and base of support. Analysis of open field locomotion with the BBB-test revealed that all rats reached a plateau of about 11. Such a score for rats that received only the contusion and not a BMSC transplant and/or CsA treatment is in agreement with a previous publication. Our observation that BMSC-transplanted rats did not exhibit improved BBB-scores over control rats is in full concurrence with previous reports but in conflict with others.

The lack of improvements of automated walking in the open field is surprising in light of the observed improvements in specific features of locomotion, locomotor pattern, and sensorimotor performance. It appears likely that BMSC-initiated mechanisms that cause the observed improvements would also be fundamental to open field automated locomotion. Future research will need to focus on this unexpected finding and elucidate possible underlying mechanism.

We found that the presence of a BMSC transplant resulted in improved responses to mechanical and thermal stimuli to the hind paws at 8 weeks after transplantation. To our knowledge this is the first report that describes effects of BMSC transplants in the injured
adult rat spinal cord on sensory function restoration. A possible mechanism that underlies the increased sensory function is tissue sparing elicited by the BMSC transplant. We did find that rats with BMSC had increased amounts of spared tissue at the contusion site and, interestingly, this strongly correlated with functional outcomes.

Rats that received daily CsA exhibited improved outcomes in some of the motor tests but not in the sensory tests. Overall, the CsA treatment did not benefit the outcome in any of the functional tests of BMSC-transplanted rats. Previously, it has been reported that treatment with CsA supports cellular and functional repair after spinal cord injury. However, we did not observe any such functional benefits of CsA treatment in transplanted rats. At present it is unclear through which mechanism CsA treatment would influence functional gains mediated by transplanted BMSC.

The presence of a BMSC transplant increased the amount of spared tissue at the contusion site at 8 weeks post-transplantation compared to control rats. This was not observed in the other treatment groups. Importantly, correlative analysis revealed that the amount of tissue sparing was strongly associated with functional outcome in rats with a BMSC transplant. These findings revealed that BMSC-mediated tissue sparing in the contused adult rat spinal cord is intimately involved in recovery of motor and sensory function.
Summary and general discussion.
Spinal cord injury (SCI) results in nervous tissue loss and immediate functional impairments. Endogenous reparative events occur within the damaged spinal cord but generally they do not result in meaningful restoration of function. Thus, SCI-mediated loss of function is permanent and people who experience SCI may be destined to spend the remainder of their lives in a wheelchair. Approaches aimed at repairing the spinal cord anatomically and functionally are being investigated in laboratories around the world. Some of the more promising approaches are being tested in the clinic but so far none of these have emerged as one that reverses the devastating functional consequences of SCI.

One of the potential treatments that could support spinal cord repair is the transplantation of cells that are known to contribute to cellular, anatomical, and functional restoration. Bone Marrow Stromal Cells (BMSC) have received ample attention for their presumed potential to repair central nervous system (CNS) lesions. An important advantage over other cellular candidates for spinal cord repair is that BMSC can be obtained from routine bone marrow biopsies from the patient. This advantage allows for autologous transplantation in which rejection of the transplanted cells may be minimal.

The overall goal of this thesis was to investigate the potential and suitability of BMSC to repair the injured spinal cord. This was addressed in a series of experiments that focused on rat BMSC gene profiling, BMSC survival after transplantation, and their effects on tissue sparing and on locomotor and sensory function restoration. For the in vivo experiments a rat spinal cord contusion model system was employed to mimic the most frequently occurring type of human spinal cord injury. In the following section, the main results from these studies are summarized and discussed in light of what is known from the current literature and what would be necessary to achieve anatomical and functional repair of the spinal cord.

In Chapter 1, SCI and a variety of related aspects are introduced. In the United States the incidence and prevalence of SCI has been studied in depth. Annually, approximately 11,000 new cases of SCI occur in the US. The size of the group of spinal cord injured people in the US has conservatively been estimated between 250,000 and 400,000. Less is known about the incidence of SCI in Europe and The Netherlands.

Mainly because of the large variability among SCI it is difficult to define the best clinical practice. During the early phase, treatments that stabilize the patient’s health and attempt to limit the overall loss of tissue/function need to be implemented. Optimal treatment needs to be determined on a case-to-case basis. Decompression surgery with or without fixation of the spinal column may accelerate functional improvements and result in shorter hospital and rehabilitation periods. As long as proper clinical trials have not been executed the effects of the timing of decompression surgery will remain elusive.
Pharmacological treatments to limit secondary injury after the initial damage have been studied intensively. Best-known is treatment with high dose methylprednisolone (MP). The effects of MP in patients with SCI were investigated in 3 consecutive National Acute Spinal Cord Injury Studies (NASCIS). The results demonstrated that acute MP treatment resulted in neurological improvements up to 6 months after injury and, as a result of these findings, MP was the standard of care for many years. However, after a thorough review of the results from the NASCIS studies and a more comprehensive assessment of the benefits and risks involved in high dose MP-treatment, the therapeutic benefits of MP treatment have been discredited. Especially in patients with complete SCI high dose steroid treatment can lead to adverse effects such as myopathy and wound infection that may negatively influence functional outcome and in some cases may be life-threatening. Currently, most clinics have discontinued the ‘standard’ acute administration of MP after SCI.

Chapter 1 also introduces stem cells (SC) as a potential therapy for SCI. Over the last years, SC have gained attention in the field of organ repair and organogenesis. Embryonic stem cells (ESC) are the cells within the inner cell mass of the blastocyst. During development these cells are restrained to germ layers where their fate is directed. Undifferentiated SC-like cells can be found among differentiated cells of a specific tissue after birth. These cells are known as adult SC, although a better term would be ‘somatic SC’ since they are also present in children and umbilical cords. It has been reported that SC are able to cross germ layers in vitro given the right ‘induction cocktail’ of growth factors. Even though this is still profoundly debated among scientists, this presumed potential has opened many new avenues for research in different disciplines.

One of the issues that surround the use of ESC is the time point at which we harvest the cells from the embryo. Can at that time the embryo in fact be considered to be alive, to be a person? Discussions on what constitutes ‘life’ and when ‘life’ starts are often intense as they are driven by moral concerns fueled by religious and political ideas. These issues need to be addressed with respect to all opponents. Ethical issues that surround the use of SC involve possible misuse, such as with therapeutic cloning and genetic manipulation. Adult SC have less ethical barriers and are therefore interesting candidates for intervention paradigms.

Chapter 2 reviews the use of BMSCs in neuroscience in general and for repair of the injured spinal cord specifically. BMSC are mesenchymal SC-like cells that reside in the bone marrow. This particular location allows for easy harvest from the patient and, consequently, for autologous transplantation with minimal rejection. Ethical issues are also undermined as BMSC can be harvested from bone marrow from adults. Whether BMSC can transdifferentiate into cells from the neural cell lineage, or other lineages for that matter, is still unclear and debated among scientists. However, the early reports that BMSC are in fact capable of transdifferentiation have launched a plethora of investigation exploring their repair potential.
Chapter 7

Some examples are the studies on the use of BMSC for repair of the heart muscle after myocardial infarction in cardiology, osteogenesis imperfecta in orthopedics, organogenesis in internal medicine, intervertebral disc disease in neurosurgery, and stroke/ neurodegenerative diseases in neurology.

As the debate on the potential of BMSC to transdifferentiate continues, they could also be used for repair for their natural ability to produce and secrete many repair-related molecules. This ability could be benefitted from in for instance the injured spinal cord as these molecules could support cellular and anatomical repair. However, when BMSC are used for spinal cord repair without differentiation into neural cells prior to transplantation, it is possible that the injected cells differentiate in vivo into mesenchymal cell types such as fat, muscle, cartilage, or bone cells. This would be a real concern as these cells would impair the overall repair of the spinal cord. So far, reports that this in fact occurs have not been published. Also, in the in vivo studies described in this thesis we have not found any signs that this takes place.

Chapter 3 describes the gene expression profile of adult rat BMSC for which 44k whole genome rat microarrays were employed. The major goal of this study was to increase our understanding of the potential and suitability of BMSC for spinal cord repair. In addition, we aimed to assess the effects of long-term culturing on gene expression by comparing BMSC from the 3rd (P3) and 14th (P14) passage in culture. Both P3 and P14 BMSC expressed genes involved in neural developmental events such as glial differentiation and myelination, and neuronal proliferation and neurite formation, indicating the potential of BMSC to differentiate into neural lineage. BMSC also expressed genes encoding for growth factors and for proteins involved in growth factor signaling. A total of 6687 genes were expressed in P3 and in P14 BMSC, with a 97% overlap of genes expressed at a similar intensity and 3% (202 genes) either higher in P3 BMSC (159 genes) or higher in P14 BMSC (43 genes). Functional data mining by Gene Ontology (GO)-analysis revealed that 85/159 and 22/43 were annotated in the GO database. In P3 BMSC, 43 GO-classes were overrepresented with 9 involved in organ development and cell proliferation. In P14 BMSC, 2 GO-classes were overrepresented with 1 involved in organ development. Overall, our gene profiling data support the use of BMSC for neural repair. A number of genes are expressed in BMSC from which the product could support cellular repair of the spinal cord. Also, we found evidence that long-term culturing of BMSC may decrease their plastic abilities after transplantation. Further gene profiling research will be needed to improve our understanding of the nervous system repair-potential of BMSC and to possibly provide a basis for manipulation of the BMSC to increase their efficacy prior to transplantation.

One possible way by which BMSC could support spinal cord repair is by limiting the progressive tissue loss (e.g., eliciting tissue sparing) that normally occurs after the initial
damage. Importantly, to profit from this ability poor survival of BMSC after transplantation into the contusion environment is a potential limiting factor. In Chapter 4 the survival of BMSC and the effects on tissue sparing after transplantation into the contused rat spinal cord are described. We injected BMSC into the moderately contused adult rat thoracic spinal cord at 15 min (acute) and at 3, 7 and 21 days (delayed) post-injury and quantified tissue sparing and BMSC survival up to 4 weeks post-transplantation. BMSC survival within the contusion at 7 days post-transplantation was significantly higher with an acute injection (32%) and 3-days delayed injection (52%) than with a 7- or 21-days delayed injection (9% both; p<0.01). Unfortunately, BMSC presence at 28 days post-transplantation was close to 0 in all paradigms, indicating massive cell death.

In contused rats without a BMSC transplant (controls), the volume of spared tissue gradually decreased until 46% (p<0.001) of the volume of a comparable uninjured spinal cord segment at 49 days post-injury. Acute and 3-days delayed but not 7- and 21-days delayed injection of BMSC significantly improved tissue sparing, which was strongly correlated (r = 0.79 - 1.0) to BMSC survival in the first week after injection into the contusion. This set of results demonstrated that BMSC transplanted into the contused spinal cord of an adult rat survive poorly and BMSC death was greater when cells were injected delayed compared to early after contusion. The data further showed that the neuroprotective effects of BMSC transplanted into a moderate rat spinal cord contusion depend strongly on their survival during the first week post-injection; acutely injected BMSC elicit more tissue sparing than delayed injected BMSC.

The results obtained in this study to some degree change some ideas on cell-based repair approaches for the injured spinal cord. For long it was thought that delayed transplantation of cells within a contusion environment would be beneficial for transplanted cell survival and, consequently, for the effects mediated by the cells, as it would circumvent the height of the injury-initiated endogenous inflammatory and immune responses. This wisdom was generally accepted within the field of spinal cord injury/repair mainly because of its logical rationale and as long as the actual delay of cell introduction within the injury would not pass the time point where general deterioration of the spinal cord would limit or even prevent any benefits from cell transplants. Even though this idea was generally acknowledged, strong supporting quantitative evidence has been lacking. Our data indicate that it may be more beneficial to transplant BMSC early rather than late at least to benefit from their tissue sparing abilities. Further research will be necessary to determine the underlying mechanisms of the improved BMSC survival with early as opposed to delayed transplantation. Also, it remains to be determined whether this would also pertain other candidate cell types for spinal cord repair.
The goal of Chapter 5 was to improve BMSC survival after transplantation into a contusion environment. We hypothesized that reducing macrophage infiltration prior to introduction of BMSC would improve their survival in the contused thoracic adult rat spinal cord. Quickly after a contusive impact to the spinal cord an invasion of macrophages can be observed. We demonstrated that treatment regimes of cyclosporine (CsA), minocycline (MC), or methylprednisolone (MP) resulted in a significant decrease (p<0.001) in macrophage infiltration at three days post-injury. Despite this promising result, survival of BMSC (1x10^6/5 μl) injected into the contusion epicenter at this 3-day time point was not significantly different from that of BMSC injected into animals without the macrophage-reducing treatments. In fact, the presence of BMSC resulted in a significant average 3.9-fold increase (p<0.001) in macrophage infiltration into the contusion of treated rats relative to controls. One conclusion from these results is that BMSC injected within the contusion epicenter attract additional inflammatory cells even with concurrent administration of macrophage-reducing drugs. It may be that those BMSC that die soon after injection recruit these extra macrophages. Death of transplanted BMSC is a multi-factorial problem which should be addressed in a multidisciplinary manner. Future studies should be directed towards finding and subsequently manipulating other cues for cell death such as pathways in necrosis or apoptosis of the transplanted cells.

In Chapter 6 we investigated the effects of BMSC grafts on hindlimb locomotor and sensory function in adult rats with a contused thoracic spinal cord. Rats were contused using the Infinite Horizons impactor at a force of 200 kDyn and 5 μl DMEM with 1x10^5 BMSCs or DMEM alone was injected into the injury epicenter three days later. Some rats received CsA daily throughout the survival period. Even though it was shown that CsA treatment did not improve BMSC survival, its administration could support repair-related events that together with those of BMSC could result in improved outcomes. Two months after BMSC transplantation rats had increased Basso-Beattie-Bresnahan (BBB) sub-scores, a 17% smaller base of support of the hind limbs, a 36% smaller angle of hind paw rotation, and performed 38% better on the horizontal ladder. In addition, at 4 weeks post-transplantation, these rats exhibited a 31% and 50% improvement in their response to a mechanical and thermal stimulus, respectively, to the hind paws. At 8 weeks post-transplantation, the rats still exhibited a 22% improvement in their response to a mechanical stimulus but not any longer to a thermal stimulus. Rats that received CsA treatment alone did not demonstrate functional changes compared to controls except for a 29% smaller angle of hind paw rotation. There was no overall additive effect of CsA treatment on BMSC-mediated functional improvements. Our data revealed that transplantation of BMSC into a 3-day old contusion in the adult rat spinal cord improved some aspects of locomotor and sensory function. Our results support further exploration of BMSC for the development of spinal cord repair strategies.
This thesis may serve as a starting point of many studies that focus on understanding and improving the efficacy of BMSC to repair the injured spinal cord. The studies in this thesis demonstrate that BMSC have the genetic ability to be effective in nervous system repair, that they elicit tissue loss which greatly depends on their survival, and that they despite their shortcomings result in behavioral improvements after spinal cord contusive injury. It is clear from our results that BMSC survival is an important issue that needs to be addressed such that their effects on repair can be optimized.
Nederlandse samenvatting.
Letsel van het ruggenmerg kan resulteren in functieverlies van de extremiteiten. Indien het verlies van functie permanent is, kunnen patiënten voor de rest van hun leven rolstoelgebonden zijn. Interventie strategiën zijn gericht op functioneel en anatomisch herstel van het beschadigde ruggenmerg, maar hebben tot nu toe niet geleid tot functionele verbetering. Ondanks de vele onderzoeken op basaal wetenschappelijk gebied is translatie naar de kliniek voornamelijk niet mogelijk.

Een van de mogelijkheden om het beschadigd ruggenmerg te herstellen is door middel van de transplantatie van cellen. Bone Marrow Stromal Cellen (BMSC) zijn stamcellen die gemakkelijk te verkrijgen zijn middels beenmergpuncties. Hierdoor is autologe celtransplantatie mogelijk en is het risico op afstoting door het lichaam minimaal.

In dit proefschrift is de geschiktheid van BMSC voor herstel van het beschadigd ruggenmerg bestudeerd. Een serie experimenten zijn uitgevoerd, waarin de genexpressie van BMSC, overleving van de cellen na transplantatie en hun invloed op de functionele uitkomst is bestudeerd in een ruggenmergletsel model in de rat.

In Hoofdstuk 1 wordt een algemeen overzicht van ruggenmergletsel geïntroduceerd. In de Verenigde Staten zijn de incidentie en prevalentie van ruggenmergletsel uitgebreid onderzocht met een incidentie van ongeveer 11,000 patiënten per jaar. De meest voorkomende oorzaken zijn ongevallen, geweld, sport en ziekte.

Wetenschappelijk bewijs voor de beste behandelmethode is er niet, mede vanwege de grote variabiliteit van de ongevallen. In de vroege fase is de behandeling gericht op hemodynamische stabilisatie van de patiënt. Hierna komt de behandeling van de (instabiele) wervelkolom aan de orde, waarbij een decompresseive laminectomie al dan niet gecombineerd met fixatie van het instabiele segment van de wervelkolom het functioneel herstel kan bevorderen en kan resulteren in een korter verblijf in het ziekenhuis en revalidatiekliniek. In de chronische fase staan preventie van infecties en doorligplekken centraal, waarbij aspecten als pijn en infertilititeit ook in ogenschouw genomen dienen te worden. Betere klinische studies zijn noodzakelijk om het voordeel van een operatie in de vroege fase adequaat te bestuderen.

De laatste jaren is het gebruik van stamcellen (SC) in de wetenschap sterk toegenomen. Embryonale stamcellen (ESC) zijn cellen in de zich ontwikkelende blastocyste. Al vroeg in de ontwikkeling bevinden deze SC zich in de kiemlagen ecto-/meso- en endoderm. Na de geboorte zijn stamcellen ook aanwezig in verschillende organen. Verschillende publicaties hebben aangetoond dat met een juist inductie protocol SC in staat zijn zich te ontwikkelen tot cellijnen uit andere kiemlagen, hoewel hiervoor nog geen consensus is bereikt. Hierdoor is er een nieuwe golf van interesse voor het gebruik van SC ontstaan.
Het belangrijkste argument tegen het gebruik van stamcellen is de discussie over wanneer een embryo een menselijk persoon genoemd kan worden. Is het begin van het leven bij de bevruchting van de eicel of bij levensvatbaarheid van de foetus? Dit is een discussie vol emotie. Potentieel misbruik van stamcellen is de voornaamste oorzaak van de vele ethische discussies.

In Hoofdstuk 2 is de beschikbare literatuurkennis over de mogelijkheden van BMSC voor herstel van het beschadigd ruggenmerg bestudeerd. BMSC zijn mesenchymale SC die in het beenmerg aanwezig zijn. Hierdoor zijn ze gemakkelijk te verkrijgen middels beenmergspunctie. Door de mogelijkheid van autografting is het risico op een afstotingsreactie geminimaliseerd. Tenslotte zijn er bij het gebruik van volwassen stamcellen geen ethische bezwaren, aangezien er geen embryonaal weefsel wordt gebruikt. Doordat verschillende publicaties hebben aangetoond dat BMSC kunnen transdifferentiëren, is er de laatste jaren toenemend aandacht hiervoor ontstaan op het gebied van herstel van de hartspier na myocardinfarcten in de cardiologie, osteogenesis imperfecta in de orthopedie, organogenese in de interne geneeskunde, neurodegeneratieve aandoeningen in de neurologie en gehernieerde discus intervertebralis in de neurochirurgie.

Door hun mesodermale origine zijn BMSC eerder geneigd te differentiëren in celtypen van de betreffende kiemlaag. Het is onzeker of deze cellen in een omgeving van het ruggenmerg niet zullen differentiëren in vet, bot, kraakbeen of spierweefsel. Het is noodzakelijk de geschiktheid van BMSC voor herstel van het beschadigd ruggenmerg goed te onderzoeken eer dit in een klinische setting te testen.

In Hoofdstuk 3 worden de resultaten beschreven van onderzoek naar de genexpressie van BMSC met behulp van 44k totaal ratgenoom microarrays. Tot nog toe is differentiatie van celtypen, waaronder ook BMSC, beoordeeld aan de hand van morfologie en membraanmarkers. Deze zijn echter niet specifiek en soms zelfs onbetrouwbaar. Het bepalen van het genexpressie patroon van BMSC is een betere manier om de geschiktheid van dit celtype voor neurale differentiatie te bepalen. Daarnaast is ook het effect van langdurige celkweken op de genexpressie van BMSC bestudeerd door passage (P) 3 en P14 te vergelijken. Zowel P3 als P14 BMSC brengen verscheidene genen tot expressie die een rol spelen in neur(on)ale processen zoals gliale differentiatie en myelinisatie, neuronale proliferatie en neurietformatie. De genexpressie van deze genen bevestigd de potentie van BMSC om te transdifferentiëren in de neurale cellijn. In totaal komen 6687 genen tot expressie met een 97% overlap in genexpressie. Van de overige 3 % komen 159 genen hoger tot expressie in P3 en 43 genen hoger in P14. Functionele data structurering laat een overrepresentatie van 43 GO-klassen in P3 BMSC zien, waarvan 9 een rol spelen in orgaan ontwikkeling en celproliferatie. In P14 BMSC zijn slechts 2 GO-klassen overgerepresenteerd,
waarbij 1 een rol speelt in orgaanontwikkeling. De resultaten steunen de potentie van BMSC als geschikte kandidaten voor verder onderzoek op het gebied van de neurowetenschappen. Tevens blijkt uit de studie dat langdurige celkweken de plasticiteit van de cellen doet afnemen.

Hoofdstuk 4 beschrijft de overleving van BMSC na transplantatie in het beschadigde ruggenmerg van de rat. Op vroege (15 min) en late (3, 7, 28 dagen) tijdstippen na ruggenmergletsel zijn BMSC getransplanteerd in het epicentrum van de laesie en is celoverleving en weefselverlies geanalyseerd tot 4 weken na celtransplantatie. Celoverleving is hoger 7 dagen na contusioneel ruggenmergletsel als de cellen direct (32%) of 3 dagen (52%) na het initiële trauma zijn geïnjecteerd. vergeleken met een injectie op 7 dagen of 21 dagen na initiële trauma (9% beiden, p< 0,01) is dit verschil significant. Helaas zijn 28 dagen na celtransplantatie op alle tijdpunten bijna geen BMSC meer te traceren, mogelijkerwijs door massale celdood.

In het contusiemodel zonder BMSC transplantatie (controle rat) neemt het volume gespaard weefsel geleidelijk af tot 46% (P< 0.001) vergeleken met een onbeschadigd ruggenmerg op een vergelijkbaar segment in de rat. Directe en BMSC-injectie na 3 dagen, maar niet injectie na 7 of 21 dagen, leiden tot een significante toename van het gespaarde ruggenmergvolume, met een sterke correlatie (r = 0.79 - 1.0) tot BMSC overleving gedurende de eerste week.

Het doel van Hoofdstuk 5 was om BMSC overleving te verbeteren in het beschadigd ruggenmerg, door de invasie van macrofagen na het initiële trauma te reduceren alvorens BMSC te transplanteren. Behandeling met cyclosporine (CsA), minocycline (MC) of methylprednisolon (MP) resulteert in een significante afname (p<0,001) van macrofaag infiltratie 3 dagen na initiële letsel. Ondanks dit veelbelovende resultaat, is er bij BMSC transplantatie 3 dagen na het initiële letsel, een vergelijkbare celoverleving tussen de behandelde en controle groep (saline injectie) na 7 dagen. De aanwezigheid van BMSC alleen zorgt voor een 3.9 maal toename van macrofaag infiltratie in het beschadigde ruggenmerg vergeleken met controle ratten, ondanks de toediening van immunosuppressiva.

In Hoofdstuk 6 wordt beschreven of het bewezen neuroprotectief effect van de BMSC de functionele uitkomst beïnvloedt ondanks de massale celdood na transplantatie. Vergeleken met controle ratten blijken BMSC tot 2 maanden na transplantatie te resulteren in een verhoogde Basso-Beattie-Bresnahan (BBB) subscore, met een verbeterde stand en rotatie van de onderste ledematen, waarbij de ratten 36% beter functioneerden op de horizontale laddertest. Vier weken na transplantatie is bij de BMSC getransplanteerde groep een 50% verbeterde responsie op mechanische en thermische stimuli vergeleken met de controle.
groep. Acht weken na celtransplantatie hadden de behandelde ratten nog een 22% verbeterde response op mechanische stimuli maar niet meer op thermische stimuli. Er is geen toegevoegde waarde van toediening van CsA op BMSC-gemedieerde functionele uitkomst. Transplantatie van BMSC in een 3-dagen oude contusie van het ruggenmerg van de rat leidt tot enig herstel in sensomotore functie. Verder onderzoek is nodig om het effect van BMSC transplantatie te optimaliseren.
Synthesis.
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Curriculum vitae
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The last sentence I want to use to dedicate this work to my late mother, who supported me throughout my life and enthusiastically supported my plans for this thesis. Unfortunately, she left us before her time, but her influences are stronger then ever; I owe you everything.
Chapter 9

Curriculum Vitae:
Rishi was born on May 2, 1978 at Zevenaar, The Netherlands. His parents moved back to Suriname at the age of 2 where he lived until his sixth year. After returning to The Netherlands he completed secondary school at Alfrink College (Zoetermeer, 1990-1996), and went on to study Medicine at Leiden University Medical Center (LUMC, 1996-2003). During this period he was active in several committees and as a teaching assistant in the dissection room. It was there where his passion for surgery started and was combined to his pre-existing interest in the nervous system. Prof. Dr. Enrico Marani (LUMC) helped Rishi to contact Dr. Martin Oudega who invited him in his laboratory at The Miami Project to Cure Paralysis to investigate the pathophysiological consequences of spinal cord injury in a rat model. In 2000-2001 Rishi worked in Dr. Oudega’s laboratory in Miami. He received a grant from the Fulbright/Netherlands America Foundation for this research project. After his return to The Netherlands he received the KNMG Dick Held Junior Research Award for the best graduation thesis at LUMC that year. Rishi finished his internships cum laude and graduated from medical school in December 2003. From January 2004 onwards he joined the Department of Neurosurgery at the Radboud University Medical Center Nijmegen, in a combined residency-PhD program (AGIKO). He received a grant from the Netherlands Institute for Sciences (N.W.O.) for his research studies which brought him in 2005-2006 for a basic research period in Dr. Oudega’s laboratory at The Miami Project to Cure Paralysis to Miami (Miami University, 2005) and at the International Center for Spinal Cord Injury (Johns Hopkins University, 2006). Rishi also spend time at the Netherlands Institute for Neurosciences (Amsterdam, 2006-2007) under supervision of Prof. Dr. Joost Verhaagen. In March 2007, Rishi returned to his residency program which he is expected to finish in March 2011 under supervision of his promotor Prof. dr. J.A. Grotenhuis. His interests in Neurosurgery are directed towards complex spinal surgery and endoscopic neurosurgery.
### Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASIA</td>
<td>American Spinal cord Injury Association</td>
</tr>
<tr>
<td>BBB</td>
<td>Basso-Beattie-Bresnahan test</td>
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<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<td>BMSC</td>
<td>bone marrow stromal cells</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosinemonophosphate</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immune sorbent assay</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FIM</td>
<td>functional independence measure</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GDNF</td>
<td>glial cell-line derived neurotrophic factor</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage - colony stimulating factor</td>
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<tr>
<td>GLGT</td>
<td>germ line gene therapy</td>
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<tr>
<td>GO</td>
<td>gene ontology</td>
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<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>iPS</td>
<td>induced pluripotent cells</td>
</tr>
<tr>
<td>LEMS</td>
<td>lower extremity motor score (ASIA)</td>
</tr>
<tr>
<td>LV</td>
<td>lentiviral</td>
</tr>
<tr>
<td>MAP-2</td>
<td>microtubule associated protein 2</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin-basic protein</td>
</tr>
<tr>
<td>MC</td>
<td>minocycline</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection (ratio of infectious virus particles to cells)</td>
</tr>
<tr>
<td>MPSS</td>
<td>methulprednisolone sodium succinate</td>
</tr>
<tr>
<td>mV</td>
<td>mili Volt</td>
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<tr>
<td>NeuN</td>
<td>neuronal marker N</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
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</tbody>
</table>
NGF  nerve growth factor
NGS  normal goat serum
NSC  neural stem cells
NSE  neuron specific enolase
NT-3  neurotrophin 3
NYU-imp  New York University - impactor (for contusion injury)
P  passage
PB  phosphate buffer
QIF  quadriplegic index of function
RAG  regeneration associated gene
RIP  rat insulin promoter
RNA  ribonucleic acid
SC  stem cell
SCI  spinal cord injury
SD  standard deviation
SEM  standard error of the mean
T  thoracic
VEGF  vascular endothelial growth factor
WISCI  walking index for spinal cord injury
List of publications:

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I DIED WAITING FOR EMBRYONIC STEM CELL RESEARCH TO FIND A CURE. WHAT ABOUT YOU?

I WAS THE EMBRYO

LET'S PRAY FOR THE SINNERS WHO ENGAGE IN THE DEVIL'S WORK OF STEM-CELL RESEARCH!

LORD, HELP THEM FIND A CURE FOR DAD'S ALZHEIMER'S...

...A CURE FOR MOM'S PARKINSON'S...

...A CURE FOR MY CANCER...

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