

## REVIEW

### Bone Marrow Stromal Cells for Repair of the Spinal Cord: Towards Clinical Application

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Stem cells have been recognized and intensively studied for their potential use in restorative approaches for degenerative diseases and traumatic injuries. In the central nervous system (CNS), stem cell-based strategies have been proposed to replace lost neurons in degenerative diseases such as Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Lou Gehrig's disease), or to replace lost oligodendrocytes in demyelinating diseases such as multiple sclerosis. Stem cells have also been implicated in repair of the adult spinal cord. An impact to the spinal cord results in immediate damage to tissue including blood vessels, causing loss of neurons, astrocytes, and oligodendrocytes. In time, more tissue nearby or away from the injury site is lost due to secondary injury. In case of relatively minor damage to the cord some return of function can be observed, but in most cases the neurological loss is permanent. This review will focus on in vitro and in vivo studies on the use of bone marrow stromal cells (BMSCs), a heterogeneous cell population that includes mesenchymal stem cells, for repair of the spinal cord in experimental injury models and their potential for human application. To optimally benefit from BMSCs for repair of the spinal cord it is imperative to develop in vitro techniques that will generate the desired cell type and/or a large enough number for in vivo transplantation approaches. We will also assess the potential and possible pitfalls for use of BMSCs in humans and ongoing clinical trials.

Key words: Bone marrow stromal cell (BMSC); Spinal cord injury; Implantation; Regeneration; Clinical trial

## INTRODUCTION

Stem cells are defined by their capacity for self-renewal and differentiation into different cell types (87, 89,94). In the early embryonic phase, stem cells are totipotent but after a few divisions the cells 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 (10,45,66,67,71).

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 exten-

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sively 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 (82) 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 (82,98,104). Interestingly, this percentage of overlap increased twofold when the C17.2 NSC clone was cultured as a neurosphere, reflecting a shift from a “stem-like” to a “differentiated” gene expression pattern (82).

The stroma of bone marrow houses multipotent cells that can differentiate into lineages of blood cells, stromal and skeletal tissue (24,25,33,58). It has been reported that these stem cells can also transdifferentiate into liver cells (84), skeletal (29,108) and cardiac (62,79) muscle cells, and CNS cells (6,14,29,48,53,66,91), but this is still debated [among others (17,109)]. 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. In addition, their ability to differentiate or transdifferentiate is unclear due mainly to the large variety of induction protocols used by different groups. For example (see also below), one group reported that about 1% of human and mouse BMSCs can be induced into the neural lineage (91), whereas another group using a different induction protocol reported that 80% of human and rat BMSCs could become neural cells (113). 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 do not attempt to fur-

ther 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 coworkers (82) 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 (82).

### HARVEST AND CULTURING OF BMSCs

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 usually removed from 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, nonadherent cells, mainly red blood cells that have a short life span of about 72 h in these culture conditions, are removed, and the remaining cells washed and further cultured in the same medium. Usually within 2 weeks after initiation, the cultures consist of spindle-shaped cells with some monocytes and macrophages present (6,60). 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 (118). Several other surface antigens (i.e., SH2, SH3, CD71, CD120a, and CD124) have been described for rat BMSCs (116).

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 dedifferentiation 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 BMSCs 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  $\beta$ III-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 [see (56) vs. (113)]. 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.

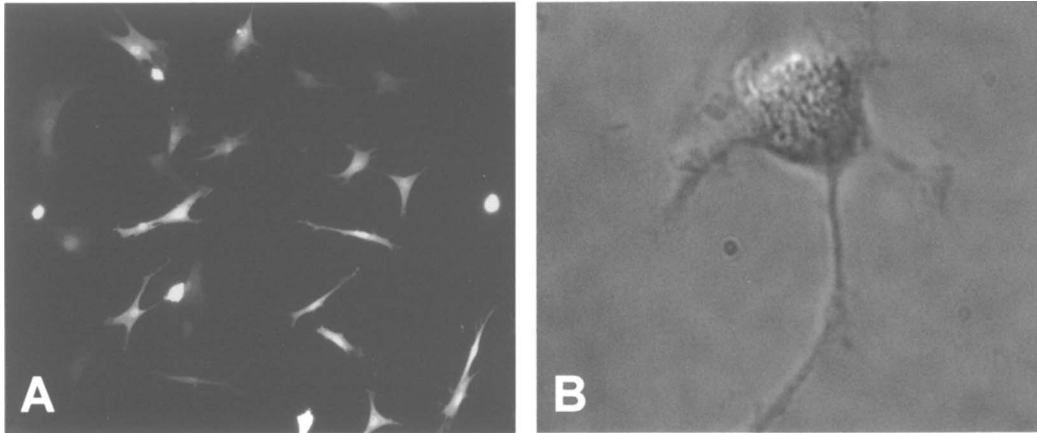
Several groups have reported that BMSCs can differentiate into cells that express neuronal markers or into cells that have a neuron-like morphology (14,67,68,80,91,92). Figure 1A demonstrates rat BMSCs isolated and cultured according to earlier described methods (6). When brain-derived neurotrophic factor (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 coworkers (80) demonstrated that human BMSCs proliferated best and expressed the highest percentage of  $\beta$ 3-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 approximately 48% (80). BDNF or neurotrophin-3 (NT-3) elicited the expression of  $\beta$ 3-tubulin up to over 40% of the cells, which could not be further increased by combining them with FGF-2 (80). 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 (80). 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 (80) may suggest that serum is necessary for neural induction, nestin-positive neural precursor cells were found in serum-free culture conditions (111). These nestin-positive cells differentiated into GFAP-positive astrocytes (based on morphology; ~40% of the population) or NeuN-positive neuronal-like cells (~19%) after 5 days in coculture with cerebellar granule cells (112). 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.

A number of studies have shown that BMSCs can be induced to become neural-like cells in vitro by adding growth factors (2,44,72,73,80,90,91,100), dibutyryl cAMP (26), or chemical agents as  $\beta$ -mercaptoethanol and dimethyl sulfoxide in combination with butylated hydroxyanisole (11,113,114). 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 differentially 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 (80) 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



**Figure 1.** (A) Undifferentiated rat BMSCs 7 days in culture expressing green fluorescent protein (GFP). The cells were isolated and cultured according to a previously described protocol (6) and infected with lentiviral vectors encoding GFP. Addition of brain-derived neurotrophic factor pushes the BMSCs into neural-like cells (B).

(13,58). 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 (56) demonstrated that the neuron-like cells derived from BMSCs by adding  $\beta$ -mercaptoethanol to the culture medium (11,113,114) 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 (56) 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  $\beta$ -mercaptoethanol. Clearly, neural cells obtained in vitro from BMSCs need to be functionally characterized. In the case of BMSC-derived neurons, demonstrating appropriate electrophysiological behavior is crucial.

#### ELECTROPHYSIOLOGICAL ACTIVITY OF BMSC-DERIVED 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 (41,42,48,112). Kohyama and colleagues (48) demonstrated that such cells exhibited a resting membrane potential ( $V_{\text{rest}}$ ) of  $-20$  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  $V_{\text{rest}}$  resembling that

of neurons, which is approximately  $-70$  mV. Jiang and colleagues (41,42) cultured BMSC-derived neuron-like cells long term with different mitogens and cytokines, then cocultured them with fetal mouse brain astrocytes and demonstrated that the neuronal-like cells had a  $V_{\text{rest}}$  between  $-8.4$  and  $-55.4$  mV. These authors also demonstrated that prolonged coculture with the fetal astrocytes resulted in a further decrease of the negative resting membrane potentials. Moreover, these cells were then able to fire action potentials (41,42). Regrettably, this study did not investigate the potential of these cells to fire trains of action potentials, a characteristic of fully matured neurons (16).

Electrophysiologically active cells derived from BMSCs were also described by Wislet-Gendebien and colleagues (112). 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  $V_{\text{rest}}$  of about  $-37$  mV. These characteristics correspond with those described for neurons in stage 1 of their maturation (16). Wislet-Gendebien and colleagues (112) further showed that after 7–15 days in culture the cells were able to fire a single-spike action potential and had acquired  $V_{\text{rest}}$  of  $-56$  mV [characteristics that corresponded to neurons in stage 2 of their maturation (16)]. 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 coworkers (112) could not demonstrate the presence of fully mature neurons [stage 3 (16)], which are able to fire trains of spikes and exhibit a normal  $V_{\text{rest}}$  of  $-70$  mV. The results from the studies mentioned above indicate that in vitro the

BMSC-derived neuronal-like cells acquire a more negative  $V_{\text{rest}}$  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 BMSCs 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 (67). Using a mouse 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 (23) 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 in vitro (105,117). Whereas this is a real possibility, Cogle and coworkers (23) 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.

### BMSCs 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 (15,88). 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 NT-3 instigates differentiation of the BMSCs into neural-like cells (80) rather than affecting survival. In vivo, in a rat contusion injury model, Hofstetter and colleagues (36) showed that more BMSCs survived when transplanted 1 week after injury compared to immediately after injury. The surviving cells were located within trabeculae that span the injury site (22,36). However, with the 1-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 low (36).

It has been proposed that one of the mechanisms underlying death of cells transplanted into the spinal cord is injury-induced inflammation (103). The cellular and

**Table 1.** Literature on Transplantation of BMSCs In Vivo in Spinal Cord Injury Models

Authors (Reference)	Source	In Vitro Modified	Injury Model	Number of Cells	Main Results	Additional Results
Chopp et al. (22)	rat	no	Contusion at T9 level. Cells grafted 7 dpi. Survival 5 weeks.	250,000	Significantly improved motor outcome at 2 weeks.	Upregulation of nestin in ependymal and associated cell layers.
Hofstetter et al. (36)	rat	no	Contusion at T9 level. Cells grafted immediate or 7 dpi, 2 mm rostral and caudal. Survival 5 weeks.	300,000	Transplanted BMSCs form guiding strands at the injury site.	Better BMSC survival with delayed grafting (7 dpi). Motor outcome improved at 5 weeks.
Akiyama et al. (3)	mice	no	EB-X lesion* T10 level. Cells grafted 3 dpi. Rats were immunosuppressed. Survival 3 weeks.	5,000	Remyelination of demyelinated axons after BMSCs transplantation.	
Corti et al. (24)	mice	no	Cells injected in tail vein after X-irradiation. Survival 3 months.	10,000,000	Systemically infused BMSCs migrate towards spinal cord.	
Inoue et al. (40)	rat	no	EB-X lesion* lumbar spinal cord. Cells grafted 3 dpi, systemic or focal. Survival 3 weeks.	100,000–10,000,000	Remyelination of demyelinated axons after BMSCs transplantation.	More myelinated axons with focal injection. Focal application more efficient.
Lee et al. (51)	mice	no	Contusion at T11 level. Cells grafted 7 dpi in and 2 mm rostrally to injury. Survival 4 weeks.	3,000	Neural differentiation: BMSCs differentiate into neurons in the brain and astrocytes in the cord.	
Wu et al. (115)	rat	no	Contusion injury T9 level. Cells grafted immediately. Survival 1, 2, 3, 4 weeks.	1,000,000	Neuroprotective: significant reduction of cavity volume.	BMSCs survival decreased in time up to 3 weeks. Motor outcome significantly improved up to 14 dpi.
Ohta et al. (77)	rat	no	Contusion injury T9 level. Immediate cell grafting in CSF (4th ventricle). Survival 5 weeks.	5,000,000	Neuroprotective: reduction of cavity volume with 47%.	Significant improvement of motor outcome after 5 weeks. BMSCs migrated to cord.
Ankeny et al. (4)	rat	no	Contusion injury T9 level. 2 dpi transplantation of cells into lesion cavity. Survival 8 weeks.	60,000	Neuroprotective: BMSCs reduce cavity volume. Increased spared tissue volume and white matter.	No significant improvement in motor outcome after 8 weeks.

**Table 1.** Continued

Authors (Reference)	Source	In Vitro Modified	Injury Model	Number of Cells	Main Results	Additional Results
Lu et al. (55)	rat	no	BMSCs-NT3 into lesioned dorsal column 5 days after cAMP in L4 DRG and NT3 immediate rostral to graft. Survival 3 months.	200,000	Axonal regeneration: cAMP and NT-3 combined promoted axonal growth of sensory axons.	
Satake et al. (93)	rat	no	Contusion T9–10 level. 3, 5, 7 dpi cell injection lumbar subarachnoid space.	1,000,000	Homing of transplanted cells towards lesion area.	Some BMSCs differentiated into nestin-positive immature neurons or glial cells.
Zurita et al. (119)	rat	no	Contusion at T7 level. Cells grafted 3 months postinjury at lesion site. Survival 4 weeks.	1,000,000	Significant improvement in motor outcome (BBB) after 2 weeks in the chronically injured rat.	BMSCs survive and form bridges in the cavity.
Sigurdsson et al. (95)	human	no	In ovo surgery and cell implantation in chicken embryo (stage 15–16). Survival 4–9 days.	20,000	Grafted cells indistinguishable from neurons chick and also electrophys. active.	During differentiation loss of CD34 expression by BMSCs.
Lu et al. (57)	rat	yes†	Dorsal column lesion C3. Survival up to 3 months.	100,000	Neural induced cells in vitro do not express neural markers in vivo.	BDNF secreting BMSCs led to higher axon density. No change in motor outcome at 3 months.

\*EB-X lesion = ethylbromide injection with X-irradiation to create a demyelinating injury.

†BMSCs were neurally induced following the procedure described by Woodbury et al. (114) and modified to express BDNF.

molecular components of the inflammatory response could initiate cell death (9), 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 provided (119). 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) (31,116), nerve growth factor (NGF) (31,50,

60), BDNF (20,50,60), and, albeit in smaller amounts, FGF-2 (60). 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 action (116). 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 BMSCs (74) 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 upregulation of apoptotic molecules or promoting the expression of antiapoptotic molecules.

### *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 (5)]. CXCL12 has been implicated in cell migration, possibly through the extracellular signal-regulated kinase (ERK) and Akt phosphorylation pathways (12,30). Interestingly, under some pathological conditions reactive astrocytes produce CXCL12 (12,69). It is possible that following spinal cord damage upregulation of the level of CXCL12 attracts CXCR4-positive BMSCs 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 (51,54,59,73), but the results regarding homing towards the injured spinal cord have been conflicting. Recently, <sup>111</sup>In-oxine-labeled BMSCs were shown to migrate poorly towards the injured spinal cord following intravenous administration (25). 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 (101). Satake and coworkers (93) 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 (25). 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 cells into the injured spinal cord is typically applied to generate a growth response, a neuroprotective effect can also be observed (102). Repeatedly, it has been demonstrated that cellular grafts limit the loss of nervous tissue in the injured cord (86,102). In fact, in animal models of spinal cord injury, improvements in motor performance seen after cell transplantation are often contributed 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 (3,4,77).

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 GDNF (31,116), NGF (31,50,60), BDNF (20,50,60), and FGF-2 (60). These factors have all been implicated in neuroprotective effects. NGF and BDNF increase survival (32,106) and decrease apoptotic death of neurons and oligodendrocytes (97). BDNF also increases oligodendrocyte proliferation (64). GDNF has been implicated in the rescue of motor neurons (8,21), possibly by activating MAP kinase and Bcl-2, an antiapoptotic regulator (21). FGF-2 is known to positively affect tissue sparing (43,65) and promote neuronal survival and angiogenesis (65) following spinal cord injury. Another molecule produced by BMSCs that could positively influence tissue sparing is VEGF, a potent angiogenic factor (34).

### *Axonal Regeneration*

In a few studies the axonal regeneration-promoting abilities of BMSCs have been addressed. Lu and coworkers (57) 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 (11, 113,114), which, as had been already recognized by Lu



and colleagues (56), causes BMSCs to die rather than become neuron-like cells.

An alternative explanation for the lower axonal growth response observed by Lu et al. (57) 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 (57,59). In another study, a multifaceted and intriguing spinal cord injury/regeneration model was used to investigate the regeneration-promoting capacity of BMSCs (55). BMSCs modified to secrete NT-3 were transplanted in a transection injury of the midthoracic dorsal columns 1 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 (55). 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 colleagues (55) 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 earlier (75) as well as in other types of axonal regeneration responses (83). 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 acutely (115), subacutely (22), and chronically (119) 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 coordination (7). 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 studies (22,115,119) 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 mechanisms (3,4,77), possibly through secretion of growth factors rather than axonal regeneration responses (55–57), 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 cervical (57) or thoracic spinal cord (4,36,115), or subacutely (22) or chronically (119) in the thoracic spinal cord. Most models involved a contusion injury (4,22,36,115, 119), others a partial transection model (57). 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 postinjury 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 as to 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 is the case, may establish BMSC grafting as the type of intervention best suited for a particular type of injury.

### CLINICAL APPLICATION OF BMSCs

There is considerable experience with the harvesting of BMSCs from the iliac crests of patients (46). In the clinic, following chemotherapy and/or radiation therapy, the bone marrow microenvironment is damaged, resulting in diminished or delayed hematopoiesis (27,63). 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 transplantation (96,99). Also, BMSCs are more increasingly used for surgical approaches for spinal fusion or for degenerated disc disease (70,110).

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 anesthetic (46), (b) human BMSCs can be rapidly and extensively expanded in cell culture (6,14), (c) there is no evidence that they produce tumors *in vivo*, even after immortalization to ensure an unlimited source of self-renewal *ex vivo* (1,28), (d) they have demonstrated capacity for tissue repair (3,57), and (e) they secrete growth factors *in vivo* that can enhance regeneration/repair (19,20). Clearly, BMSCs may be a good candidate for transplantation into the injured spinal cord. However, a concern is the considerable variation existing among donors such as gender, genetic background, and general health (74). Therefore, we agree with Neuhuber and colleagues (74) 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 (52), but also efficacy trials in osteogenesis imperfecta (18,37,

76), Hurler's syndrome and metachromatic leukodystrophy (47). 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 US Food and Drug Administration (FDA) require safety data prior to efficacy data. Consequently, most cellular transplantation strategies tested in clinical trials in the US 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 beneficial. Clinical trials transplanting BMSCs after spinal cord injury are ongoing in several countries, including Korea, Mexico, Colombia, and Brazil. Other than a Korean study (81), results from these trials have not yet been published. 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 during the first 2 weeks after injury and a total of 1.8 ml with a density of  $1.1 \times 10^6$  BMSCs/ $\mu$ l was injected at the epicenter of a contusion lesion at 5 mm below the dorsal surface. In five out of the six patients motor and sensory function improved, with four of the patients 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 [e.g., (91) vs. (113)]. This leads to a great deal of confusion on the true capacity of these cells and thus their therapeutic potential. There is a clear 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 stan-

dardized criteria [operational definition (82)] that will result in the proper designation of BMSC-derived cells as neurons or oligodendrocytes. Only then will it be possible to optimally benefit from the therapeutic potential of BMSCs for spinal cord repair.

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 whereto? 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 in vitro and 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 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 in vivo have shown promise in the laboratory and clinical trials are ongoing.

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