Cell. Mol. Life Sci. 63 (2006) 1649–1657
1420-682X/06/141649-9
DOI 10.1007/s00018-006-6019-5

Cellular and Molecular Life Sciences

Review

Coaxing bone marrow stromal mesenchymal stem cells towards neuronal differentiation: progress and uncertainties

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Received 19 January 2006; received after revision 24 February 2006; accepted 12 April 2006
Online First 19 June 2006

Abstract. Multipotent adult stem cells capable of developing into particular neuronal cell types have great potential for autologous cell replacement therapy for central nervous system neurodegenerative disorders and traumatic injury. Bone marrow-derived stromal mesenchymal stem cells (BMSCs) appear to be attractive starting materials. One question is whether BMSCs could be coaxed to differentiate *in vitro* along neuronal or glial lineages that would aid their functional integration post-transplantation, while reducing the risk of malignant transformation. Recent works suggest that BMSCs could indeed be differentiated *in vitro* to exhibit some cellular and physiological characteristics of neural cell lineages, but it is not likely to be achievable with simple chemical treatments. We discussed recent findings pertaining to efforts in neuronal differentiation of BMSCs *in vitro*, and results obtained when these were transplanted *in vivo.*

Keywords: Bone marrow, differentiation, glia, mesenchymal stem cells, neuron.

Introduction

As the use of pluripotent human embryonic stem (ES) cells poses multiple ethical problems [1] and because it is still unclear whether patient-specific human ES cells could be produced [2], the development of therapeutic replacement strategies using adult stem cells remains a very realistic option. Bone marrow stromal cells (BMSCs) have been of great interest in this regard as a source of autologous stem cells for cell replacement therapy for central nervous system (CNS) neurodegenerative disorders and traumatic injury. This interest is founded partly on the availability of relatively simple clinical procedures for harvesting bone marrow aspirate and BMSCs' high capacity for self-propagation in culture. BMSCs are progenitors of various cells of mesodermal origin, such as skeletal tissue components and adipocytes [3]. However, one major factor for therapeutic interest in BMSCs is their apparent ability to differentiate into cells of other developmental lineages [4] – of particular interest here would be those of the neuroectodermal lineage.

The ability of BMSCs to differentiate into neural cells when transplanted into brain was initially demonstrated by Blau's laboratory [5] as well as Mezey and colleagues [6–7]. This phenomenon, however, was not apparent according to others [8–9]. It was subsequently found that the transdifferentiation phenomenon associated with BMSCs could be accounted for by fusion with local cells [10–11]. This was found to occur not just for BMSCs transplanted into brain, but in liver [12–13] and other tissues as well [14]. While cell fusion could clearly explain a good fraction of the apparent neural transdifferentiation of transplanted BMSCs, there continues to be new evidence to suggest that, at least in some particular experimental paradigms, transdifferentiation does indeed occur [15–17].

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Initial studies of transplanting BMSCs into animal stroke [18–20], spinal cord injury [21–23], amyotrophic lateral sclerosis [24] and Parkinson's disease [25–26] models indicated some beneficial effects. These studies, taken together, attest to the clinical potential of BMSC-based transplantation therapy. It has been argued that the field should push forward to evaluate the therapeutic potential and end points in the use of these cells, despite uncertainties and our lack of understanding of the physiological behavior and developmental fate of transplanted BMSCs [27]. One aspect of BMSC research has been numerous attempts to drive its differentiation towards neural fate *in vitro* prior to transplantation. The ability to manipulate BMSCs *in vitro* serves two logical purposes. First, pre-differentiation of BMSCs into more restricted neural cell types could enhance its ability to anatomically and functionally integrate into particular parts of the brain, with presumably higher efficacy in ameliorating lesions that are peculiar to specific disorders. Second, differentiated neural cells (especially when they become post-mitotic) are generally considered to have a lower chance of malignant transformation compared with actively dividing precursors.

In the past 5 years or so, there have been many reported attempts to differentiate BMSCs derived from multiple mammalian species *in vitro* into neurons (or cells that exhibit neuronal phenotypes). Some of these involved rather simple chemicals, while others made use of growth factor cocktails. Still others have more elaborate schemes involving transgenic expression of particular genes. These reports are categorized and summarized in Table 1, and we briefly discuss them below with comments on their relative merits. It should be kept in mind that BMSCs are by nature a heterogeneous population of cells with subpopulations of different morphology, proliferative capacity and multipotency. Due to the lack of particular stem cell markers for BMSCs, it is often not possible to clearly distinguish one subpopulation from another in terms of ability to differentiate, or transdifferentiate.

Potential problems in the use of simple chemicals for *in vitro* neural differentiation of BMSCs

A rather wide range of chemicals have been used in attempts to initiate neural differentiation of BMSCs. Deng et al. [28] attempted to elevate cellular cyclic AMP (cAMP) levels of human BMSCs using membrane-permeable dibutyryl cAMP (dbcAMP) in conjunction with isobutylmethylxantine (IBMX), a phosphodiesterase inhibitor, and reported the appearance of neuron-like cells constituting about 25% of the population after 6 days. Woodbury et al. [29–30] pioneered a three-step protocol where cells were preincubated in medium containing β-mercaptoethanol (BME), induced with serum-free medium containing an antioxidant, butylated hydroxyanisole (BHA) and dimethyl sulfoxide (DMSO), and maintained in a complex maintenance medium containing the above plus valproic acid, forskolin (a stimulator of adenylated cyclase) and N2 supplement. Both protocols showed an apparent induction in the neuronal marker neuron-specific nuclear protein (NeuN), with nestin also elevated after 5 h in the Woodbury protocol. A particularly remarkable feature of the Woodbury protocol is the rapid onset of morphological changes and NeuN expression, which occurred in a matter of hours. The protocol also apparently resulted in higher degrees of cell death compared with Deng's when assessed in parallel [31]. However, the induced population remained alive in the Woodbury maintenance medium, losing nestin but gaining TrkA expression when examined after 6 days.

Recent findings have cast some serious doubts on whether the use of these simple chemicals, particularly those employed in the Woodbury protocol, could actually result in neural differentiation [32–34]. There was an initial suspicion that the phenomenon may be caused, or at least accompanied, by cellular events other than differentiation. First, the morphological changes did not come with clear observations of neurite growth cones, and were associated with a high degree of cell death. Second, and more important, this apparent transdifferentiation occurred in a seemingly unrealistic time frame of hours, compared with the days required for BMSCs to differentiate into cells of mesodermal origin. In these later reports, it became apparent that changes in the cell morphology that resembled neuronal differentiation could in fact be due to cell shrinkage and loss of focal contacts resulting from disruption of the actin cytoskeleton. Treatment with low pH or high salt produced similar morphological changes [32], which could also be observed when other cell types, such as fibroblasts, were subjected to BHA/DMSO treatment [32–34]. Conversely, a plethora of cell stressors such as EDTA, cytocalasin D and detergents could also elicit similar changes in BMSCs. Although the expression of classical neuronal markers, NeuN and the 200-kDa neurofilament (NF-200), was judged to have increased based on immunostaining observations, these appeared to be aberrantly localized in the cell, and elevation of protein levels could not be confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Another point to note is that BMSCs appear to already express varying levels of neural and astrocytic markers, and inhibition of protein synthesis with cycloheximide did not prevent cells from adopting 'neuron-like' morphology after chemical induction. This apparent lack of a need for transcriptional or translational change undermines the suspected 'trans-differentiation' nature of the observed changes.

In a more in-depth probe of changes in the transcriptome, Bertani et al. [34] showed by a 21,000-gene microarray analysis of BMSC cells 6 h and 48 h after induction that the BHA/DMSO induction protocol has little effect on the

Table 1: In vitro neuronal/glial differentiation of BMSCs.

Treatment	Starting material	Verification of neuronal/glial phenotype	References
Chemicals			
Isobutylmethylxantine (IBMX) + dibutyryl cAMP	human BMSCs	neuron-like morphology, expression of neuronal markers determined by WB	Deng et al. (2000) [28] Rismanchi et al. (2003) [31]
β-Mercaptoethanol (BME) followed by butylated hydroxyanisole (BHA) and dimethylsulfoxide (DMSO)	rat BMSCs	neuron-like morphology, expression of neuronal markers determined by WB and IHC	Woodbury et al. (2000, 2002) [29–30] Munoz-Elias et al. (2003) [16] Rismanchi et al. (2003) [31] Jori et al. (2005) [72]
5-Azacytidine	clonal lines derived from murine BMSCs	neuron-like morphology, expression of neuronal markers determined by RT-PCR and IHC, electrophysiological characterization and calcium uptake imaging	Kohyama et al. (2001) [38]
β-Mercaptoethanol (BME) + retinoic acid (RA)	sized-sieved Human BMSCs	neuron-like morphology, expression of neuronal markers determined by WB and IHC, electrophysiological characterization	Hung et al. (2002) [35]
Butylated hydroxyanisole (BHA) + dibutyryl cAMP + isobutylmethylxantine (IBMX) + retinoic acid (RA) + ascorbic acid	mouse BMSCs	expression of NSE and NeuN	Levy et al. (2003) [73] Hellmann et al. (in press) [61]
Neurotrophic (or glial) factors/gene transfection			
Combination of GDNF, PACAP and dibutyryl cAMP	sized-sieved BMSCs	neuron-like morphology, expression of neuronal markers determined by WB and IHC	Tzeng et al. (2004) [74]
Generation of neurospheres by culturing with bFGF/EGF and induction with RA and Sonic hedgehog	mouse BMSCs	neuron-like morphology, expression of neuronal markers determined by WB and IHC	Locatelli et al. (2003) [54]
Transfection with the Notch intracellular domain (NICD) followed by treatment with neurotrophic factors (such as BDNF)	human, mouse and rat BMSCs	neuron-like morphology, expression of neuronal markers determined by WB and IHC, electrophysiological characterization	Dezawa et al. (2004) [25] Kamada et al. (2005) [62]
Transfection with noggin	clonal line-derived from murine BMSCs	neuron-like morphology, expression of neuronal markers determined by IHC, calcium uptake imaging	Kohyama et al. (2001) [38]
Glial growth factor (GGF)	rat BMSCs	cells exposed to GGF-expressed GFAP and S100	Tohill et al. (2004) [61]
Human neural stem cell (NSC) culture conditions	human BMSCs	formation of nuerosphere-like aggregates with expression of proneural genes	Hermann et al. (2004) [55]
Co-culture with neural cells			
Co-culture with rat or mouse fetal midbrain cultures	human and mouse BMSCs	morphology and IHC labeling of neuronal markers	Sanchez-Ramos et al. (2000) [50]
Co-culture with astrocytes	murine bone marrow multipotent adult progenitor cells (MAPCs)	neuronal morphology, expression of neuronal markers determined by IHC, electrophysiological characterizations	Jiang et al. (2002, 2003) [4, 52]
Co-culture with mouse NSCs	rat BMSCs	nestin-positive cells selected in classical NSC medium could differentiate into GFAP- positive expressing astroglial-like cells when co-cultured with mouse NSCs	Wislet-Gendebien et al. (2003) [56]
Co-culture with cerebellar granule neurons	rat BMSCs	nestin-positive cells selected in classical NSC medium could differentiate into GFAP-positive astroglial-like cells or NeuN-positive and electrically excitable neuron-like cells when co-cultured with mouse NSCs	Wislet-Gendebien et al. (2005) [59]

overall transcription profile of the population. The treatment also did not significantly change the levels of certain neural-associated markers which the BMSCs are already expressing; neither did it affect other neural markers. The set of genes whose levels did change with the treatment intersected very poorly with the ones that are differentially expressed between the BMSCs and fetal brain. In fact, the intersection is just as poor with genes that are differentially expressed between the BMSCs and adult liver. These new findings suggest that the morphological changes resembling neural differentiation observed with the induction protocol could have largely resulted from a fast-onset cellular response to chemical toxicity, and not a differentiation program that should be dependent on significant changes in the transcriptome. Obviously, in spite of a possible burst of cell death, the population as a whole eventually survived, and components added in the maintenance medium may have helped. Since there is an absence of the common neuronal markers, it is unclear what phenotype this population assumed after 6 days.

In another, somewhat similar approach, Hung et al. [35] used a 3-μm filter to size-select a homogeneous population of human BMSCs. These size-sieved BMSCs were morphologically different from those described above (which are large, flat cells to begin with), being more fibroblastic in appearance. When subjected to preincubation in medium with BME (with or without retinoic acid) followed by serum deprivation, morphological changes resembling neural differentiation occurred within 2–3 h in > 95% of the cells, but which apparently reverted back to the cells' original morphology after 5 h. These already expressed a significant amount of β3-tubulin (TuJ), with detectable NeuN, nestin and neuron-specific enolase (NSE). There were moderate elevations in nestin, NeuN, tau and NSE proteins after 5 h. Remarkably, cells that were serum-deprived for 5 days lost TuJ and NeuN expression, and showed instead expression of neurofilament, but not the dendritic marker microtubule-associated protein-2 (MAP-2). The extent of cell death in the 5-day culture was not described, but appeared not to be particularly extensive from the Hoeschst (DNA dye) staining presented. These cells exhibited voltage-sensitive ionic currents, and intracellular calcium levels could be elevated by a high K^+ buffer and glutamate in the medium. In this case, it is also unlikely that the morphological changes observed in 2–3 h represents true neuronal differentiation, but some degree of differentiation may have taken place over the time course of 5 days, with the cells kept alive by the neuroprotective effects of BME. Again, despite some electrical excitability, a lack of common neuronal markers made it difficult to tell what identity these cells had eventually assumed. It should be noted, however, that in other reports, BMSCs first treated with BME/retinoic acid and subsequently cultured in the presence of forskolin, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and heregulin assumed a morphology similar to Schwann cells, and expressed Schwann cell markers such as p75, glial fibrillary acidic protein (GFAP), S-100, O4 and P0 [36–37].

A notable chemical-based variation to the above protocols involved the use of 5-azacytidine (a demethylating agent). Kohyama et al. [38] isolated clonal lines from murine BMSCs, which were also fibroblastic in appearance. About 20% of these could be gradually (~9 days) differentiated into cells morphologically resembling neurons with 5-azacytidine in medium supplemented with a neurotrophin cocktail of nerve growth factor (NGF), neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF). Interestingly, these cells expressed both neuronal (such as TuJ and NeuN) as well as glial [such as galactosylceramidase (Gal-C) and GFAP] markers, with each line having a different percentage of neuronal versus glial marker expression. In addition, after 28 days of induction, the cells exhibited a resting potential, a rectifying K^+ current and could respond to neurotransmitters. These cells, therefore, appeared to have been differentiated to some extent along the neural lineage.

Particular transgene expression could enhance neural differentiation

Kohyama et al. [38] also devised another method to effect *in vitro* neural differentiation of their BMSC lines. The product of *Noggin* acts antagonistically to bone morphogenetic proteins (BMPs), promoting neurogenesis and suppresses glial differentiation [39]. When one of the BMSC lines named KUSA/A1 (which appeared to be particularly osteogenic) was transfected with *Noggin*, cells detached and formed neurosphere-like aggregates. Upon plating on ornithine/fibronectin-coated dishes, these cells exhibited a neuron-like morphology, with ~50% in the population expressing the post-mitotic neuron marker MAP2, and only ~5% expressing GFAP. These cells also exhibited calcium influx when stimulated with high K^+ buffer and glutamate. Although further characterization of the Noggin-expressing cell line is necessary to assess its neurogenic capacity, the result suggested that Noggin may in this case act both in a cell autonomous and non-autonomous way to enhance neural differentiation. More recent results have also indicated that when BMP signaling is repressed by Noggin in cultured human ES cells, non-neural differentiation is suppressed and the cells also develop into neurosphere-like aggregates highly enriched in neural progenitors [40–41]. Transgenic expression of Noggin, or culturing BMSCs in a defined medium with Noggin, may be worth further investigation in so far as BMSC neural differentiation *in vitro* is concerned.

An intriguing recent report of trangene expression-enhanced BMSC differentiation involved the use of the Notch

intracellular domain (NICD). Notch is a large transmembrane protein with widespread function in development, and has long been known to be important in shaping the nervous system [42] and function in cell fate determination [43–44]. Upon activation by its transmembrane ligands Delta/Serrate/Lag-1, Notch undergoes regulated intramembrane cleavage (RIP) [45] and an intracellular fragment (the NICD) is translocated into the nucleus where it modulates transcriptional control of differentiation factors. Dezawa and colleagues [25] reported the creation of NICD-transfected BMSCs, which could be driven by a cocktail of neurotrophic factors [a combination of bFGF, ciliary neurotrophic factor (CNTF) and forskolin] into neuron-like cells after 5 days. These cells have a resting potential of −50 to −60 mV and demonstrated an outwardly rectified K^+ current. The most remarkable feature of these cells appeared to be mitotically terminated, and they expressed the post-mitotic neuronal marker MAP-2. Further treatment of these cells with glial-derived neurotrophic factor (GDNF) could in fact generate tyrosinase-positive cells that could secrete dopamine.

Further investigation into the possible mechanism of action of NICD in BMSCs suggested the involvement of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway [46–47]. A JAK/STAT signaling inhibitor induced a significant amount of MAP-2 positive cells in neurotrophic factor treated non-NICD-transfected BMSCs, whereas STAT3 alleviated the effect of NICD when co-transfected. On the other hand, overexpression of known NICD-activated basic helix-loop-helix (bHLH) transcription factors Hes1 and Hes5 did not mimic the NICD effect [25]. This is perhaps in line with the fact that Hes1 and Hes5 are repressor-type bHLH factors that suppress neurogenic activities of the activator class of bHLH factors, such as Mash1, Math and Neurogenin [48]. It is unclear if NICD could indeed induce Hes1 and Hes5 in BMSCs. Notch induction of Hes1 and Hes5 are known to be essential for the maintenance and proliferation of neural stem cells [48–49]. Expression of NICD in BMSCs may have resulted in reprogramming of its transcriptome to perhaps a state that bears some similarity to neural progenitors. This point is speculative at the moment, but a full-scale gene expression profiling analysis of NICD-expressing BMSCs (compared with untransfected, as well as before and after neurotrophic factor treatment) could be revealing, and important in understanding the effect of the transgene expression at the molecular level.

Co-culturing of BMSCs with neural cells enhances neural transdifferentiation *in vitro*

A number of studies have indicated that BMSCs could be first 'tuned' towards neuronal differentiation with a cocktail of growth factors and then attain full neuronal characteristics in co-culture with neurons or astrocytes. Sanchez-Ramos et al. [50] showed that a small fraction of the large, flat human and mouse BMSCs cultured in epidermal growth factor (EGF) or retinoic acid/BDNF expressed nestin, NeuN or GFAP. The proportion of NeuN-expressing cells increased when BMSCs were co-cultured with rat fetal midbrain cells, but cells with distinct MAP-2 expression were never observed.

The BMSC populations discussed above are largely cells from bone marrow aspirates that have adhered and survived multiple passages. Most of them appeared as relatively homogenous large, flat cells (some may assume a more spindle shape when confluent), and are typically CD34 and CD45 negative but CD90/Thy-1 positive. These are also largely osteogenic, adipogenic and chondrogenic. Therefore, although their intrinsic 'stemness' is not easily quantifiable, they are clearly multipotent. On the other hand, the bone marrow aspirate also appears to contain a rare subpopulation of cells that behave rather more like ES or neural stem cells, although their relationship with the BMSC cells discussed above is not quite clear. These cells, termed multipotent adult progenitor cells (MAPCs) by Verfaillie and colleagues, could be isolated and selected based on surface immunogenic markers [4, 51–52] and propagated extensively without senescence *in vitro*. Reminiscent of ES and neural stem cells, rodent, but not human MAPCs require leukemia inhibitory factor (LIF) (in addition to PDGF and EGF) for *in vitro* expansion and maintenance [4, 52]. Again reminiscent of neural stem cells, removal of EGF and PDGF with the addition of bFGF to mouse MAPCs induced, after 14 days, neuronal or glial marker expression in >90% of the cells (~70% expressing NF-200). Nestin expression came on as early as day 5. A more elaborate sequential treatment with bFGF, Sonic hedgehog (Shh)/FGF-8 and BDNF generated more mature neuronal phenotypes with dopaminergic (dopadecarboxylase/tyrosine hydroxylase), serotonergic (serotonin) and γ-aminobutyric acid (GABA)-ergic neuron markers. Polarity and maturity of the neuronal phenotype were also shown by domain-specific tau and MAP2 expression.

In another recent report, Kondo et al. [53] showed that BMSCs constitutively express components of the Shh pathway and retinoic acid nuclear receptors. Interestingly, a combination of RA and Shh (but not any of these alone) synergistically induced the expression of a set of glutamatergic sensory neuron markers in BMSCs primed with FGF2 and forskolin. In addition, embryonic day 10 hindbrain/somite/otocyst-conditioned medium or prenatal cochlea explants promoted similar upregulation of sensory neuron markers and process outgrowth. These interesting results suggest that a proper mimic of environmental factors could potentially lead BMSC differentiation into particular neuronal subtypes.

Adult astrocytes from hippocampus are capable of regulating neurogenesis of neural stem cells by apparently instructing the stem cells to adopt a neuronal fate [54]. While astrocytic conditioned medium did not further enhance the neuronal phenotype of the treated MAPCs above, co-culturing with fetal (E16) brain astrocytes did promote a more mature neuronal morphology with more elaborated axons. Importantly, while astrocytic conditioned medium-treated MAPCs and co-cultured MAPCs are electrically excitable and exhibited an outward current, only the latter exhibited spiking associated with an inward current that was blocked by tetrodoxin. This indicated the expression of functional voltage-gated sodium channels, a hallmark of mature neurons.

Other workers have also attempted to derive stem cell-like populations from BMSCs [20, 55–58]. Locatelli et al. [55] and Hermann et al. [56] observed the formation of neurosphere-like cell aggregates that were nestin-positive after 5–7 days in culture medium containing bFGF and EGF. In the latter report, these human neural stem cells derived from BMSCs can be differentiated *in vitro* into glia and neurons. Differentiated neurons exhibited dopamine production and release as well as outward and inward currents. Rogister and colleagues [57] made similar observations with what they termed rat stromal cells or mesenchymal stem cells (MSCs). They surmised that serum removal is important for induction of nestin expression, and that nestin expression is a pre-condition for the formation of neurosphere-like cell aggregates and progression towards further neuronal differentiation. Interestingly, co-culturing of nestin-positive MSCs with neural stem cells resulted in the formation of heterogenous spheres, which when plated out on a substratum exhibited a high degree of GFAP expression, but no observable neuronal marker expression. In fact, these MSCs appeared to be able to stimulate astroglial differentiation of the neural stem cells by releasing BMP-4 [59]. However, when MSCs were co-cultured with cerebellar granule neurons [60], they expressed neuronal markers, exhibited both outward and inward currents and were responsive to neurotransmitters. The observations described above clearly attested to the ability of BMSCs to be converted to a population that is more committed to a neural fate *in vitro*. They further suggested that the subsequent neural cell fate of transplanted BMSCs *in vivo* would be largely determined by the environments in which they are engrafted, especially influences exerted via cell-to-cell contact.

Concluding remarks: survival and function of *in vitro* 'differentiated' BMSCs *in vivo*

There are indications that somewhat pre-selected BMSCs could differentiate into various neural cell types and ameliorate neurological defects associated with experimental stroke [20]. Likewise, NICD-expressing BMSCs that are BDNF-treated and tyrosinase positive significantly improve behavioral indices associated with the 6-hydroxydopamine (6-OHDA) rat model of Parkinson's disease [25]. Interestingly, BMSCs pre-treated with bFGF and EGF and subsequently 'induced' by a cocktail of BHA, dbcAMP, AMP, IBMX, retinoic acid and ascorbic acid more than survived engraftment in a 6-OHDA model [61]. These cells in fact seemed to survive better in the damaged hemisphere compared with undamaged side, and engrafted cells appeared to have subsequently migrated from the left striatum through the corpus callosum to 6-OHDA-lesioned right striatum. It was unclear whether the survival and migration of engrafted BMSCs were associated with any improvement in behavior.

It should also be kept in mind that functional and behavioral recovery need not be due to the formation and integration of functional neurons. Engrafted BMSCs could potentially improve recovery in several ways. For example, BMSCs differentiated to assume a Schwann cell-like morphology by a combination of BME/retinoic acid and growth factor treatments [36, 62] stimulated sciatic nerve regeneration. These cells had also been shown to significantly improve BBB scores when incorporated in a Matrigel-based transplant in a rat spinal cord transaction model [63]. Along the same line of thought, it may be good enough, at least at the first instance, if engraftments function in a neuroprotective manner to preserve surviving neurons, enhancing their survival and possibly regeneration [64]. In view of the above, the optimal level of *in vitro* manipulation would necessarily be disease or lesion-specific, and would by and large constitute a balance between how helpful the extent of prior differentiation would be for functional integration versus survival and neuroprotective benefits. While fully differentiated, dopaminergic neurons would be preferred in the treatment of Parkinson's disease, transplantation therapy for traumatic CNS injuries may not benefit from the use of fully differentiated neurons.

Another point to note is that *in vitro* manipulation involving transgene expression, no matter how good the rationale is to begin with, should be carefully evaluated in animal models for potential drawbacks and side effects. While transplantation of multineurotrophin-expressing glial-restricted precursor seems to aid the recovery from traumatic spinal cord injury [65], a Noggin-expressing stem cell graft (which would supposedly neutralize neurogenesis inhibiting BMPs at the injured spinal cord) increased both lesion volume and macrophage infiltration [66], despite earlier evidence to the contrary [67].

At the present moment, methods of differentiation and assessments of *in vitro* differentiated BMSCs and their subsequent behavior *in vivo* have remained rather fragmented. Most studies are done using heterogenous cell

populations with different development potentials. This heterogeneity complicates interpretation of results. For proper development of therapeutic approaches, a meticulous and systematic approach is necessary to work out optimum protocols that would be reproducible and reliable in generating transplantable BMSCs from patients' bone marrow aspirates. Thus, methods for the selection and expansion of BMSCs should be further developed. We should continue to explore conditions for in vitro differentiation [68], and future analysis would benefit from the use of better-characterized cell populations and clonal cells. It would appear that we could realistically aim to achieve the ability to generate equivalents of neural stem cells from BMSCs, or to be able to manipulate these like neural stem cells. In order to do this, we need to know more about the BMSCs before we can develop methods for efficient clonal selection, expansion, characterization and *in vitro* differentiation. A more comprehensive understanding of the transcriptome (particularly changes in relevant gene families [69]), the proteome and the molecular cell biology (such as the role of cell cycle checkpoint regulators [70]) of BMSCs before, during and after differentiation is clearly necessary. Finally, continuous exploration of how BMSCs survive and develop in various animal transplantation models [61, 71] is also vital. Only with a repertoire of multifaceted and detailed information could we hope to acquire molecular handles, in the form of combinatorial and temporally expressed markers, that would allow effective *in vitro* and *in vivo* manipulations of these cells.

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