

# Integral Therapeutic Potential of Bone Marrow Mesenchymal Stem Cells

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**Abstract:** Bone marrow derived mesenchymal stem cells (MSC) are adult stem cells that reside within the bone marrow compartment. In the traditional developmental model, adult stem cells are able to differentiate only to the tissue in which they reside. Recent data have challenged the committed fate of the adult stem cells, presenting evidence for their multi-lineage differentiation potential. In addition, potential therapeutic benefits of MSC administration have been the main concern of much research, including clinical trials. These studies promote adult stem cell therapy by shedding some light on the therapeutic potential of MSC and their mechanism of action.

Many doubts have found their way into MSC research. They question MSC potency and beneficial contribution. However, these obstacles should not arrest but set a challenge to MSC researchers to examine their achievements under a magnifying glass.

Therapeutic benefits of MSC exogenous delivery do not run counter to its possible participation in endogenous repair. Several reports imply MSC involvement in physiological repair but no explicit data support this hypothesis.

This review tries to put MSC research into perspective. Possible therapeutic applications of MSC therapy for damaged tissue replacement, tissue engineering and the underlying repair mechanisms will be discussed. In addition, reported data about MSC possible involvement in physiological multiple tissue repair, their homing to injury and site-specific differentiation will be presented.

**Key Words:** Mesenchymal stem cells, Homing, Differentiation, Tissue regeneration, Stem cell therapy.

## INTRODUCTION

Stem cell therapy presents a promising and innovative technique for tissue repair and regeneration, partly imaginary and partly real. It represents great expectations, promising results and many doubts. Different therapeutic strategies, based on stem cells have been developed and studied.

The most primitive of all stem cell populations, offering the most potential, are embryonic stem cells (ESC) that are obtained from the inner cell mass of developing blastocysts. ESC are pluripotent stem cells that can give rise to cells of the three germ layers found in the implanted embryo, fetus, or developed organism, but not to embryonic components of the trophoblast and placenta. Applications of the embryonic pluripotent stem cells to clinical studies, have been impeded due to: potential immune rejection in allogeneic transplantation [1], formation of teratomas [2], lack of their availability and serious ethical and political issues [3]. In view of the latter, autologous cell sources may prove to be more beneficial and acceptable as a therapeutic tool in the future.

Adult stem cells are found in different tissues of the adult organism and remain in an undifferentiated, or unspecialized, state. They possess the ability to self renew, and can differentiate into at least one mature, specialized cell type. In

the traditional developmental paradigm, adult stem cells are able to differentiate only to the tissue in which they reside. Recent data challenge the committed fate of the adult stem cells and present evidence for their plasticity. Thus, adult stem cell therapy may offer an accessible, therapeutic tool for damaged tissue replacement and tissue engineering that is free of ethical debate.

The plasticity of the adult stem cells may provide treatment for a broad spectrum of diseases, especially neurodegenerative disorders and brain injury. In these conditions the best therapeutic methods are limited due to their inability to repair or to replace the damaged tissue. Traditionally, the mammalian central nervous system (CNS) was considered to be a nonrenewable tissue, but this principle has been challenged in the past decade. Studies have demonstrated that neural stem cells (NSC) exist not only in the developing mammalian nervous system but also in the adult nervous system of all mammalian organisms, including humans [4, 5]. NSC are capable of undergoing expansion and differentiation into neurons, astrocytes, and oligodendrocytes *in vitro* [6, 7] and after transplantation *in vivo* [8]. However, inaccessibility of NSC sources deep in the brain severely limits their clinical effectiveness. As to endogenous brain repair, neurogenesis in adult brain is restricted to certain brain regions, lessens with age and does not allow significant functional recovery of the damaged brain tissue. For that reason, neuronal death and functionally damaged or completely dysfunctional remaining tissue are the consequences of brain injury or neurodegenerative disease [9]. Various therapeutic

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strategies aim to protect the remaining neurons, to slow down the progression of damage, to replace the damaged tissue and to reduce the severity of the symptoms. Efficient neuroprotection has yet to be achieved and since the symptoms of most brain injuries and neurodegenerative diseases occur after major neuronal loss, neuroprotective benefits are limited. Moreover, pharmaceutical therapy is only partly effective, since it cannot replace the lost neurons or stop the progression of the damage and usually causes serious side effects with prolonged use. These limitations are not unique to neurodegenerative diseases and brain injuries, as in many diseases injured tissue repair or regeneration cannot be achieved and only symptomatic therapy is provided.

Here, we review the integral therapeutic potential of bone marrow (BM) mesenchymal stem cells (MSC), a BM-derived type of adult stem cell. Reported data about the possible involvement of MSC in physiological multiple tissue repair, their migration and site-specific differentiation, will be discussed. We will also present MSC differentiation potential *in vitro*, their benefits to regeneration and therapy of nervous system and other tissues and the problems arising in adult stem cell research.

## MESENCHYMAL STEM CELLS - HISTORY AND DEFINITION

Over thirty years ago Friedenstein *et al.* [10-12] defined fibroblast-colony-forming-cells from the BM that adhere to cell culture surfaces and can differentiate into osteoblasts, adipocytes and chondrocytes - mesenchymal cell types, *ex vivo* and *in vivo*. In later experiments, fibroblast-colony-forming-cells were termed MSC or BM stromal cells (BMSC) [13, 14]. In the following years, most of the researches concentrated on the BM stroma created microenvironment, which is essential for lineage commitment and differentiation of hematopoietic stem cells and has regulatory roles in hemopoiesis [15, 16]. Much later studies hypothesized that after induced differentiation, MSC may acquire a phenotype distinct from that of the precursor. If so, adult BM is an available source of autologous stem cells for treatment of many common and currently incurable disorders. Later studies by Jiang *et al.* [17] report an existence of a rare multipotent adult progenitor cells (MAPC) that can be obtained from human BM. MAPC can be identified in the adherent cultured MSC only after the cells undergo approximately 30 or more population doublings. The MAPC population is characterized by rapid replication and can differentiate into multiple mesenchymal cell types as well as hematopoietic lines, representing a primitive progenitor cell. To date, no research has determined whether MAPC are a small rare subpopulation of MSC or a new cell population developed during its purification.

## ISOLATION, CULTURE AND EXPANSION OF MESENCHYMAL STEM CELLS

Human MSC are isolated from a BM aspirate, which is often collected from the superior iliac crest of the pelvis. In many protocols the whole marrow sample is fractionated on a density gradient, such as Percoll, after which the cells are plated at densities ranging from  $1 \times 10^4$  cells/cm<sup>2</sup> to  $0.4 \times 10^6$  cells/cm<sup>2</sup> in growth medium. The general growth medium is

based on Dulbecco's Modified Eagle's Medium (DMEM) or  $\alpha$ -Modified Eagle's medium ( $\alpha$ -MEM) with 10% fetal bovine serum (FBS) [14, 18, 19]. Rodent BM is usually harvested by flushing femurs and tibias with the growth medium [20]. During culture, MSC adhere to plastic culture dish and display fibroblast-like morphology.

MSC represent a very small fraction of BM cells, only 0.001-0.01 % of the total population of nucleated cells in BM. However, millions of cells could be harvested after several passages of the cells in culture, with no occurrence of spontaneous differentiation [14]. Curiously, millions of MSC could be obtained from aged BMSC originated from geriatric patients. Furthermore, aged MSC could be cryopreserved at  $-80^\circ\text{C}$  without evident loss of viability, differentiation and proliferation capability [21]. These encouraging data imply that aged MSC are capable of proliferation, differentiation and may be useful for cell therapy in elderly patients.

However, culture conditions may change MSC characteristics and may reduce their therapeutic potential. Previous studies have shown that during culture expansion MSC undergo an aging process in which their early progenitor properties, proliferation and homing capability are gradually lost [22-24]. Donor age, too, has been associated with a reduced proliferative capacity of MSC. This negative effect of *in vitro* expansion and donor age on proliferative capacity of MSC has been correlated with the rate of their telomere loss. Current culture protocols that involve population expansion stimulate rapid aging of MSC, with large telomere shortening and little remaining proliferative capacity. Surprisingly, some MSC cultures were able to maintain telomere length for over 40 population doublings after an initial telomere shortening. Telomere length preservation may point toward a selection of more primitive MSC, a small population that may not always present in all samples due to the small volume of BM primary sample [25]. In contrast, MAPC, selected from BM mononuclear cells by depletion of CD45 and glycophorin-A (Gly-A) cells, can be cultured for more than 70 doublings and have long telomeres that do not shorten during culture [17, 26].

The low frequencies of MSC in the BM require their expansion *in vitro* prior to their use in cell therapy. However, the culture and its duration may harm MSC proliferation, differentiation and homing capacity and therefore need to be taken into consideration.

## SURFACE MARKERS OF MESENCHYMAL STEM CELLS

Many researches tried to characterize MSC by specific surface markers. Three detected antibodies SH2, SH3, and SH4 recognize antigens on the cell surface of BM-derived MSC and do not react with BM-derived hematopoietic cells, osteoblasts or osteocytes [27]. The SH-2 monoclonal antibody raised against human MSC reacts with endoglin (CD105), the TGF- $\beta$  receptor III that is mainly associated with endothelial tissues. It has been suggested that CD-105 may play a role in chondrogenic differentiation of MSC and in mediating interactions between hematopoietic stem cells and MSC in the BM [28]. SH-3 and SH-4 antibodies recognize different epitopes of CD-73 (ecto-59-nucleotidase)

present on the surface of MSC. CD-73 is a membrane-anchored protein with both ecto-5'-nucleotidase activity and signal transduction capability. It plays a role in the salvage of purines and pyrimidines, mediates cell-cell interactions in the bone and represents a common element of stromal and lymphocyte development [29].

STRO-1 has been identified as an antibody that reacts with the nonhematopoietic BMSC. Interestingly, flow cytometric analysis of the STRO-1 antigen expression on the BMSC showed increasing numbers of STRO-1 positive cells up to the second week of culture, followed by a progressive decline. It was suggested that the loss of STRO-1 expression in prolonged culture is related to the *in vitro* maturation of stromal precursors into more differentiated stromal cell types. However, a small number of STRO-1 positive cells remain after prolonged culture and are able to produce adherent cell layers that are indistinguishable from the parent cultures [30].

SB-10 antigen, present on undifferentiated MSC, was identified as CD166 (ALCAM). This marker disappeared after *in vitro* osteogenic differentiation of MSC [31].

CD44, another surface protein expressed on MSC is hyaluronic acid/fibronectin receptor that is involved in hematopoietic cell adhesion to BM stroma, proliferation and mobilization [32, 33].

Furthermore, MSC express CD13, CD29 (  $\alpha$ 1 integrin), CD58, CD71, CD90 (Thy-1), CD106 (VCAM-1), CD120a, CD124, and many other surface antigens. In contrast, MSC were found negative for: CD3, CD4, CD11c, CD15, CD16, CD19, CD31 (PECAM), CD33, CD34, CD38, CD56, CD62P, CD104, CD144 and hematopoietic lineage markers CD14 and CD45 [14, 32]. It is possible, however, that while the CD34 antigen is expressed by the MSC when directly isolated from the BM, it disappears during culture expansion, similar to other markers whose expression is influenced by conditions and duration of the culture.

Characterization of MSC by specific antigens will allow a different, more specific and accurate isolation method than plastic adherence, distinction of BM-derived MSC subpopulations and their relative contribution to different therapeutic effects. Unfortunately, there is no consensus about adequate markers to allow selection of purified MSC population.

### **IN VITRO DIFFERENTIATION OF MESENCHYMAL STEM CELLS**

In addition to osteoblastic, adipocytic and chondrocytic differentiation, MSC acquired an unexpected phenotype after their exposure to different factors *in vitro*. Various studies induced MSC differentiation into neurons and glial cells. After *in vitro* neural differentiation cells demonstrated an assortment of neuronal and glial markers, such as neuron-specific enolase (NSE), neuronal nuclear antigen (NeuN), neurofilament M (NF-M), tau, glial fibrillary acidic protein (GFAP) and adopted neuronal and glial morphology [34-37]. Interestingly, Woodbury *et al.* [38] found that BMSC expressed germline, ectodermal, endodermal, in addition to mesodermal, genes prior to the induced neural differentiation and hypothesized that neural differentiation involves modulated expression of these genes. After extensive research and

development numerous neural differentiation protocols were created. These protocols were based on MSC treatment with retinoic acid (RA) and epidermal growth factor (EGF) or brain-derived neurotrophic factor (BDNF) [34], fibroblast growth factor (FGF) and RA [35], isobutylmethylxanthine (IBMX) and dibutyryl cyclic AMP (dbcAMP), that elevate intracellular cAMP levels [36], butylated hydroxyanisole (BHA) and dimethyl sulfoxide (DMSO) [37, 38]. Not only neural, but dopaminergic neuronal fate was obtained by *in vitro* induced differentiation. Our laboratory demonstrated that after induced dopaminergic differentiation along with the neuronal morphology, cells expressed NeuN and activated transcription factors that are typical to midbrain dopaminergic neurons. In addition, differentiated cells expressed elevated levels of dopamine related genes - AADC, D2DR and DAT [39]. Mouse BM derived MAPC have also displayed neuroectodermal morphology and markers of mid-brain dopaminergic, serotonergic, and GABAergic neurons after sequential stimulation with basic fibroblast growth factor (bFGF), sonic hedgehog (SHH), FGF8, and BDNF. Furthermore, coculture of neuron-like differentiated cells with fetal brain astrocytes extended their survival and promoted their morphological maturation. Differentiated neuron-like cells displayed electrophysiological characteristics typical of neurons [40].

The neuroectodermal phenotype is not the only one exhibited by MSC after induced differentiation *in vitro*. For instance, MSC obtained from adult human BM were induced to differentiate into cells with a cardiomyocyte phenotype, after treatment *in vitro* with 5-azacytidine and bFGF. The differentiated cells expressed various myogenic markers and displayed functional characteristics similar to cardiomyocytes and skeletal muscle cells [41].

Physiological and pathological processes involve the formation of new blood vessels. In the past, it was thought that these blood vessels were formed due to the migration and proliferation of mature endothelial cells. However, recent findings show that the formation of new blood vessels involves circulating endothelial progenitor cells (EPC) that home to the injured site and participate in its repair. EPC have been identified both in peripheral blood and in BM. CD133 (+) hematopoietic stem cells, BM and peripheral blood derived CD34 (+) cells and peripheral blood mononuclear cells can be induced to display endothelial markers, *ex vivo* [42]. In addition, MAPC were found to differentiate into endothelial cells. These MAPC were CD34 (-), vascular endothelial cadherin (-), AC133 (+), and fetal liver kinase (+) [43]. MSC were also successfully induced to differentiate into cells with phenotypic and functional features of endothelial cells [44].

The liver too, may perhaps be regenerated by differentiated MSC. Rat MSC were stimulated to differentiate into cells with hepatocyte-like phenotypes after treatment with HGF [45].

These studies are evidence that *in vitro* treatments of MSC may induce differentiation or at least drive the expression of tissue specific markers. Cellular therapy represents two therapeutic strategies. One supports transplantation of undifferentiated cells into the tissue, hoping to achieve their integration, site specific differentiation regulated by local

signals, and if not differentiation then at least therapeutic benefits. Another approach supports differentiation, partial or maximal, of MSC *in vitro* and transplantation of the differentiated cells. Both strategies illustrate the potential therapeutic effects of MSC.

### POTENTIAL PARTICIPATION OF MESENCHYMAL STEM CELLS IN PHYSIOLOGICAL HEALING

Different studies were performed in order to find out whether BMSC can be mobilized from BM to peripheral blood, in response to artificially admitted stimulating factors or injured tissue, and if these cells contribute to the general mesenchymal tissue turnover and repair. Extended definition of mesenchymal tissue includes (myo) fibroblasts, bone, cartilage, fat, tendon, muscles and nerve tissue [46].

### Mesenchymal Stem Cells may Circulate in Peripheral Blood

The debate concerning the potential mobilization of MSC from the BM is associated with MSC's presence in normal or mobilized peripheral blood. Several studies have detected MSC in the peripheral blood. Fernandez *et al.* [47] studied fourteen female breast cancer patients who received daily recombinant human growth factors: granulocyte-colony stimulating factor (G-CSF) or granulocyte macrophage colony stimulating factor (GM-CSF) after undergoing chemotherapy. In 11/14 samples of peripheral blood progenitor cells low density mononuclear cells gave rise to an adherent layer containing fibroblast-like and large flat round cells. These cells produced collagen (I, III and VI), fibronectin, expressed adhesive ligands VCAM-1 (CD106), ICAM-1 (CD54) and expressed surface antigens recognized by monoclonal antibodies, SH2 and SH3 which had been used to characterize BM derived MSC. These cells were negative for CD34, CD45 and CD14, myeloid progenitor antigens. Researchers could not conclude whether mobilization of stromal cells from the BM occurs as a consequence of chemotherapy, growth factor administration or both.

Another study found a small population of CD34 (-) mononuclear cells that proliferated rapidly in culture and adhered to plastic and glass in the blood of 100 normal individuals. These cells were positive for vimentin, collagen I and BMP-receptors, heterodimeric structures expressed on mesenchymal lineage cells. In addition, these cells stained for CD105 surface marker of BM MSC and had a capacity to differentiate into fibroblast, osteoblast and adipocyte lineages [48].

Not all studies see eye to eye with these findings. Wexler *et al.* [49] studied stem cells obtained from the BM, the cord blood and the peripheral blood stem cell collections. The cultured cells derived from peripheral blood stem cell collections did not survive the first passage and did not produce phenotypic or morphological evidence that these were MSC. In the BM cultures a confluent layer of fibroblast-like cells with mesenchymal cells immunophenotype was observed. The possibility that MSC or their precursors could be present in mobilized adult peripheral blood at a low frequency was not excluded. However, they did not proliferate in their culture system.

### Mesenchymal Stem Cells Express Homing Related Receptors

MSC expression of migratory-related receptors supports the theory that MSC can be mobilized into peripheral blood and can participate in tissue repair. Stromal derived factor-1 (SDF-1) has been shown to be critical in promoting the migration of stem cells to the BM, *via* its specific receptor CXCR4. CXCR4-positive tissue-committed stem/progenitor cells were released/mobilized from BM into peripheral blood during organ injury and chemoattracted to the damaged peripheral tissues by SDF-1 gradient. It was suggested that these cells constitute a different from BM MSC, nonoverlapping subset of cells [50]. A recent study investigated the expression of CXCR4 receptor in human BMSC cultures. All cultures were CD45 and CD34 negative and bound the SH2 monoclonal antibody raised against human BMSC. Less than 1% of the cells showed expression of extra-cellular loop of the CXCR4 receptor. However, the expression of the intracellular CXCR4 was detected in 83-98% of the sampled cells. In a transwell assay, a MSC migration trial revealed a small proportion of MSC that strongly express the functionally active CXCR4 receptor, capable of promoting migration to the BM. The study concluded that CXCR4, although present at the surface of a small subset of BMSC, is important for mediating specific migration of these cells to the BM and mobilization of the internalized receptor may be required to improve engraftment of MSC [51]. The conclusion that SDF1/CXCR4 plays a major role in endogenous stem cell migration to injured tissues, together with the claim that BM MSC express the internalized CXCR4 receptor, may imply that endogenous MSC may home to various injured tissues and participate in their repair.

It has been reported that rat MSC express CXCR4 and CX<sub>3</sub>CR1, the respective receptors for SDF-1 and fractaline and the chemokine receptors CCR2 and CCR5. SDF-1, fractaline and their receptors were factors in MSC migration in a rat model of hypoglossal nerve injury. However, it is still unclear if endogenous rat MSC are able to home to fractaline or SDF-1 expressing sites as part of physiological tissue turnover and repair [52].

### Bone Marrow Progenitor Cells are Mobilized from the Bone Marrow and Contribute to Endogenous Tissue Repair

The theory that endogenous stimuli and exogenous cytokine therapy may mobilize progenitor cells from BM and contribute to endogenous tissue repair, has been mentioned in the literature. Exogenous GM-CSF was discovered to enhance the mobilization of EPC from the BM. In addition, the development of regional ischemia in both mice and rabbits increased the frequency of circulating EPC, characterized as SCA-1 positive cells in mice and as a population depleted of T-lymphocytes, B-lymphocytes and monocytes in rabbits. Furthermore, there is direct evidence that EPC that contributed to neovascularization, the creation of new blood vessels, were endogenously mobilized from the BM in response to ischemia and GM-CSF [53].

A different study showed that BM cells mobilized by stem cell factor (SCF) and G-CSF homed to the infarcted heart and promoted cardiac repair. The researchers suggested

that Lin negative, c-kit positive cells are responsible for the cardiac repair but their results did not provide explicit information about the origin of the cells that reconstituted the myocardium. They also proposed that it is possible that cytokine treatment mobilized BM stem cells and resident cardiac stem cells, which together participated in tissue regeneration [54].

As yet, there is a lack of evidence supporting a functional role of MSC mobilized from the BM, in endogenous repair of multiple mesenchymal tissues.

### SYSTEMICALLY DELIVERED MESENCHYMAL STEM CELL MIGRATION

Many researchers were fascinated by the fate of systemically delivered MSC. This interest gave birth to different studies that investigated the inhabitation and the distribution of systemically infused MSC. Multi-organ homing of MSC was found after intra-artery (i.a.), intravenous (i.v.) and intraperitoneal cavity (i.p.) infusions of labeled MSC into rats. After both i.a. and i.v. infusion, radioactivity associated with MSC was detected primarily in the lung and then secondarily in the liver and spleen. After i.p. infusion, small amounts of radioactivity were observed in kidneys, spleen, liver and lung. Researchers suggested that the distribution of MSC to different organs after various infusions may indicate that MSC circulate in the blood or lymphatic flow and eventually home into different organs [55].

In a different work on baboons, labeled MSC were tracked after systemic infusion. MSC were transduced *in vitro* with a green fluorescent protein (GFP) retroviral construct and infused into 3 adult baboons following lethal total body irradiation. Two baboons received autologous injection and one allogeneic injection of labeled MSC. Allogeneic and autologous MSC appeared to be distributed in a similar random manner and could be detected in a wide range of non-hematopoietic tissues including gastrointestinal tissues, kidney, skin, lung, thymus, and liver. The cells were detected 9 and 21 months after the injection and were assumingly capable of participating in a cellular turnover and replacement within an engrafted organ [56].

There is much evidence that systemically administered MSC migrate to many tissues, and not just to the BM, their endogenous residence and may be traced in these tissues after a long period of time. The signals that are responsible for this homing and the physiological significance of this migration are yet to be defined.

### SITE SPECIFIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

The outcome of the migrated MSC is another interesting issue. Proliferation of the delivered MSC *in vivo*, site specific differentiation and integration of the injected cells into the existing tissue, are still being debated. Liechty *et al.* [57] examined the destiny of the delivered MSC after systemic administration. Human MSC population was administered by intraperitoneal injection at either 65 or 85 days of gestational age of fetal sheep, before and after the expected development of immunologic competence. In this system, MSC presented a model of nonselective distribution

and lasting survival, for as long as 13 month after transplantation. Site specific differentiation of the transplanted MSC into chondrocytes, adipocytes, myocytes, cardiomyocytes, BMSC and thymic stroma was demonstrated. Long term cell engraftment was detected even when the cell injection was performed after expected development of immunologic competence. It was concluded that MSC possess multiple lineage differentiation potential and display long-term engraftment after in-utero transplantation in sheep.

Local transplantation, a different cell administration technique, has been adopted by many researchers. Using this technique, Toma *et al.* [58] studied human MSC differentiation after injection into the left ventricle of murine hearts. This study illustrated the ability of adult human MSC to integrate and to undergo striated muscle differentiation in the adult heart and supports the prospect of using these human adult stem cells for therapeutic cardiomyoplasty.

The study of Kopen *et al.* [59] explored whether BMSC acquire neural lineage phenotypes after direct injection into lateral ventricles of neonatal mice. The data show that BMSC migrated throughout the forebrain and cerebellum imitating the behavior of neural progenitor cells. Some MSC within the striatum and the molecular layer of the hippocampus expressed GFAP, implying astrocyte differentiation. Part of the injected cells within the reticular formation of the brain stem, were stained for neurofilament, suggesting neural differentiation.

An important study investigated induced differentiation of CD90 positive MSC into photoreceptors in the rat eye. Adult rat MSC were induced to express, *in vitro* photoreceptor-specific markers, rhodopsin, opsin, and recoverin. After labeling the cells with GFP or bromodeoxyuridine (BrdU) they were administered subretinally into rats' eyes. When administered to ocular microenvironment MSC underwent differentiation rather than proliferation, integrated into the retina and were potentially capable of signal transduction [60].

These studies demonstrated engraftment of administered MSC into different tissues, site specific differentiation and their long term survival. The absence of reported proliferation or foreign differentiation of the delivered MSC and their site specific differentiation may imply that MSC migration and differentiation are tightly regulated events. The key players that control these restricted processes continue to be an enigma. This is in contrast to the different reports of unforeseen teratomas after ESC transplantation, probably due to the different differentiation programs activated simultaneously.

### THERAPEUTIC BENEFITS OF MESENCHYMAL STEM CELL ADMINISTRATION

The rationale behind cell delivery to promote tissue repair is based on the belief that endogenous repair involving the mobilization of MSC from the BM, is insufficient in several pathological conditions. Therefore, cell transplantation or an enhanced mobilization of endogenous therapeutic reservoir may contribute to tissue functional recovery. Different studies have showed homing capability of artificially administered MSC and their site specific differentiation.

These findings, along with the studies that showed MSC multiple-lineage differentiation potential *in vitro*, recommends adult MSC as a very promising prospective therapy. Yet, all these potential benefits will have to prove real and beneficial in actual tissue repair. Some of the recent studies in this field will be discussed here.

### Infarcted Heart

Infarcted myocardium has captured much attention from supporters of stem cell therapy, especially since myocardial infarction is a common, potentially fatal condition, which may cause irreversible myocardial damage [61]. The study of Bittira *et al.* [62] reported that following myocardial infarction, intravenously injected MSC migrated to the injured zone and showed positive staining for cardiomyocyte and smooth muscle cell specific markers. Equally important is the finding that the cells migrated only to the territory of the infarcted myocardium while adjacent normal myocardium remained unaffected. MSC homing to the injured heart occurred within days following myocardial infarction and its limited duration may explain the incomplete healing of the infarcted myocardium. Differentiated cells may contribute to the pathophysiology of post-infarct remodeling, angiogenesis, and maturation of the scar [62].

Barbash *et al.* [63] argued the feasibility and the beneficial effects of systemically delivered MSC. One week after intravenous delivery of BM MSC into infarcted rat myocardium, the labeled cells were identified either in the infarcted or in the border zone but not in remote myocardium. In addition, these cells were identified in the lung, liver, spleen, and BM. Interestingly, most transplanted cells were trapped in the lung. It was proposed that the surgical procedure or even myocardial infarction created the lung injury that may have entrapped the infused cells. The researchers concluded that systemic delivery of MSC, is limited by lung entrapment of the donor cells.

Many studies evaluated therapeutic benefits of BMSC local transplantation to the infarcted myocardium models. A particular study injected BMSC into borders of the left ventricular wall scar tissue 4 weeks after infarction. Supporting BMSC previously reported therapeutic potential, locally delivered BMSC appeared to have beneficial effects and improved cardiac performance even after the healing process has occurred. Histological examination suggested that implanted BMSC engrafted into the myocardium and gave rise to a new myocardium and blood vessels [64].

### Skeletal Muscle

The regeneration of injured skeletal muscle is imperfect and may leave the patient with a weakened and unbalanced muscle. Since much progress with conventional treatment has not been achieved, the possible contribution of transplanted MSC has been explored. Natsu *et al.* [65] transplanted allogeneic cells termed BM-derived mesenchymal stromal cells from GFP-transgenic rats to the injured site of the skeletal muscle injury model in rat. The transplanted cells caused accelerated maturation of myofibers, restored muscle strength to nearly normal, one month after the transplantation, and advanced skeletal muscle repair. The grafted cells did not differentiate into skeletal myofibers or

fuse with them, although the possibility that they differentiated into muscle precursor cells was not rejected. Alternatively, the transplanted cells contributed to the regeneration of skeletal muscle through indirect mechanisms possibly based on interactions among the grafted cells, inflammatory cells, and muscle satellite cells.

### Central Nervous System

#### Brain Injury

Interestingly, studies were performed in order to improve functional outcome of traumatic brain injury (TBI). Since the optimal protocol for cell transplantation and the ideal amount of transplanted cells that will maximize the benefits is unknown, the study used two different doses of BMSC:  $2 \times 10^6$  cells and  $1 \times 10^6$  cells. Systemically injected BMSC successfully migrated into the rat brain following TBI, selectively concentrated around the injury site and partially expressed immature neuronal marker Tuj1 and an astrocytic marker, GFAP. Functional improvement was dose dependent, became evident on day 7 and elevated with time, with a higher dose being significantly more beneficial. Researchers, basing their suggestion on the current and previous studies, concluded that the functional recovery may be a result of neurotrophic growth factors production and not due to the damaged neurons replacement [66].

A later study of Mahmood *et al.* [67] investigated the mechanism of functional recovery after intravenous administration of rat BMSC in rat TBI model. In addition to functional outcomes, the levels of nerve growth factor (NGF), BDNF and bFGF were measured quantitatively assuming that they may be a part of the functional recovery. As in the previous study, functional improvement of cell treated rats was observed. The expression of NGF and BDNF was significantly amplified in the transplanted group and the expression of bFGF remained the same as its expression in the control group. Immunohistochemical analysis implied that the transplanted BMSC probably produced the trophic factors by themselves and induced their production in the surrounding glial tissue. Increased expression of growth factors may play an important role in functional progress in this study and in other studies as well.

Since stroke is a candidate for stem cell therapy, many studies evaluated the beneficial effects of MSC administration in stroke models and examined their mechanisms of action. In stroke, the contribution of angiogenesis to the brain capillary network, though important and sometimes even life saving cannot support potential brain functional recovery. In this case, possible therapeutic role of MSC might be to improve angiogenesis. In the rat model of stroke, human intravenously delivered MSC caused increased vascular perimeter, augmented numbers of capillaries and bigger amounts of proliferated endothelial cells. Interestingly, only about 1% of the injected MSC exhibited the endothelial phenotype. It is possible that endothelial cells from the neighboring tissues and from circulating EPC contributed to the angiogenesis. Furthermore, the transplanted MSC increased endogenous expression levels of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR2), which promote angiogenesis in the adult ischemic brain. An indirect mechanism of action was

proposed - MSC may produce a wide variety of trophic factors and cytokines from the endogenous cells, some of which activate the production of VEGF and VEGFR2 which contribute to angiogenesis and tissue repair [68].

A question yet to be answered, is what signals direct and promote the administered MSC to the ischemic tissue. *In vitro* microchemotaxis chamber assay was used in an attempt to resolve this issue. It was found that monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), interleukin-8 (IL-8) and ischemic brain tissue extracts at 24, 48 hours and 1 week after ischemia promote human MSC migration in the transwell chemotaxis assay [69].

Sensory-motor functional improvement was evident within two and six weeks after direct transplantation of human MSC into the rat brain after experimental stroke. The transplanted cells grafted into the cortex surrounding the area of the infarction and expressed different neural markers: astrocytes marker - GFAP, oligodendroglia marker - GalC, and neuronal markers - beta III, NF160, NF200, human NSE, and human NF70. However, even after six weeks the grafted MSC still maintained their primitive properties and produced collagen I and fibronectin. Collagen I and fibronectin may participate in MSC survival and differentiation, and may be involved in functional recovery by activating integrin signal transduction, and forming new neuronal circuits in host brain tissue. In this case, as in previously presented case related studies, the functional improvement was presumably mediated by proteins secreted by the transplanted MSC and not by integration of newly created neurons into existing tissue [70].

A recent study presents a new *in vivo* fluorescence optical imaging protocol to track transplanted cells in the living animals. This technology was used to follow GFP BMSC transplanted into the ipsilateral striatum of the adult mice after the onset of middle cerebral artery occlusion. The transplanted cells migrated into the ischemic boundary zone within 4 weeks; several cells displayed neuron-like morphology and were reactive for the neuronal marker MAP2. Interestingly, the survived cells in the striatum showed no significant differentiation into the neural cells. After twelve weeks, the transplanted cells were distributed in the entire brain, especially in the neocortex adjacent to the infarct. Considerable numbers of GFP-positive cells were reactive for MAP2, simulated neuron-like appearance and expressed GFAP in certain regions. Tracking the cells during their migration in the brain and the evaluation of the markers they expressed drove a conclusion that differentiation or fusion of the transplanted BMSC depends on their surrounding micro-environment [71]. This study did not evaluate functional improvement but stressed important information about the timing of the transplanted cells migration and the site specific processes that cells undergo in different brain regions. The widespread distribution of the transplanted cells warrants further study.

### Neurodegenerative Disease

Neurodegenerative diseases, such as Parkinson's disease (PD), are appropriate candidates for treatment with MSC. Current treatment options of PD are only partially effective. They do not arrest degeneration and often cause serious side

effects. Cell transplantation in PD might promote functional recovery or halt the degeneration of the remaining dopaminergic neurons. The clinical therapeutic potential of BMSC in PD was demonstrated by injecting mouse BMSC intrastrially to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. The transplanted cells survived, expressed tyrosine hydroxylase, a rate limiting enzyme in dopamine synthesis and reduced motor dysfunction. The rapid therapeutic benefit and the low numbers of MSC present in the target tissue support the assumption that production of growth factors rather than newly formed neurons, was responsible for functional recovery [72].

A recent study of stem cell transplantation for PD treatment used *in vitro* differentiated MSC after their transfection with Notch intracellular domain. Differentiated cells expressed neuronal markers and some of them displayed action potential compatible with the characteristics of functional neurons. These cells were further treated with glial cell line-derived neurotrophic factor (GDNF) that promoted dopaminergic differentiation. Transplantation of treated cells into the ipsilateral striatum in a 6-hydroxy dopamine rat PD model demonstrated behavioral recovery. In addition, dopamine release was detected in a study group, displaying significant behavioral recovery. Interestingly, this study correlated dopamine secreting differentiated MSC and functional recovery in PD model [73].

### Spinal Cord Injury

The injured brain is not the only target of the adult stem cell therapy in CNS. Different studies explored beneficial potential of BM MSC when transplanted into spinal cord injury model. In the study of Hofstetter *et al.* [74] rat MSC underwent neural differentiation, expressed mature neuronal markers but could not develop electrophysiological potential. Local injection of the treated cells one week after the injury promoted grafted cell survival and exerted a beneficial effect on functional recovery, that could not be achieved if cells were injected immediately after the injury. In fact, the delivered cells formed nerve fiber-permissive tissue bridges across the epicenter of the lesion. Immature astrocytes, nestin-positive and GFAP-negative, populated these MSC bundles. These MSC bundles were associated with and contributed to a long-term functional improvement in the injured spinal cord.

Another study infused rat BMSC into the cerebrospinal fluid (CSF) through the 4th ventricle of injured rat spinal cord. Most BMSC attached to the surface of the spinal cord but part of them invaded into the cord lesion. Both populations disappeared within 3 weeks after injection. The transplanted cells did not differentiate into GFAP-positive astrocytes, O4-positive oligodendrocytes, or NF (or tubulin)-positive neurons and still induced behavioral improvement and tissue repair of the injured spinal cord. It is possible that BMSC produced trophic factors into the CSF that promoted repair of the lesion, causing a reduction in volume of cavities that are caused by progressive tissue necrosis in the injured spinal cord [75].

Remyelinating potential of MSC was reported by the study of Akiyama *et al.* [76]. Direct injection of BMSC into the lesion area of rat spinal cord caused remyelination of

demyelinated axons and improved the conduction velocity of the remyelinated axons.

Although several studies reported the therapeutic effects of MSC in spinal cord injuries and demyelinated spinal cords, they could not determine whether the transplanted cells underwent differentiation, and newly differentiated cells promoted tissue repair or if MSC promoted an endogenous repair process. Since the precise mechanism of the repair is unknown both scenarios may be partially correct.

### PERIPHERAL NERVE INJURY

Recent study showed that BMSC might be beneficial in peripheral nerve injuries. The study of Dezawa *et al.* [77] isolated rat MSC and induced *in vitro* schwann cell differentiation that included treatment with beta-mercaptoethanol followed by RA based medium and finally cultured in the presence of forskolin, human bFGF, human platelet derived growth factor-AA (PDGF) and human heregulin-beta1 (HRG). Transplantation of the differentiated cells into sciatic nerve after sciatic nerve injury demonstrated nerve-fiber regeneration, accompanied by myelin synthesis.

Furthermore, MSC treated *in vitro* with glial growth factor had beneficial effect on schwann cell development within regenerating nerves after transplantation into a rat model of peripheral nerve injury [78].

### CLINICAL TRIALS

MSC participated in several clinical studies that were able to demonstrate the feasibility of mesenchymal cell therapy. Allogeneic BMSC labeled by neomycin phosphotransferase marker gene were injected into children with severe osteogenesis imperfecta. Transplanted BMSC integrated, differentiated into osteoblasts and fibroblasts, and produced clinical improvement. No major toxicity was detected except for an urticarial rash in one patient just after the second infusion of BMSC [79].

Several patients with Hurler's syndrome (mucopolysaccharidosis type-IH) and metachromatic leukodystrophy (MLD) have also received allogeneic injection of BM derived MSC. No toxicity was detected and MSC injection was associated with clinical benefits [80].

BMSC transplantation alone or co-infusion of MSC with hematopoietic stem cells has also proved to be beneficial in serious pathological conditions [81, 82].

The promising results of clinical studies suggest that donor allogeneic MSC infusion might be safe, feasible treatment and may be related to the reversal of disease pathophysiology in several pathologies.

### UNRESOLVED ISSUES

As previously mentioned, not all the researchers agree on the broad spectrum of MSC differentiation potentials to generate a tissue they do not reside within. It is unclear whether dedifferentiation of small population or reprogramming of MSC to a more pluripotent cell, transdifferentiation, is responsible for its multi-lineage differentiation potential. Several studies claim that multi-lineage differentiation of

MSC could be attributed to cell fusion and not to the differentiation process [83-87].

Lu *et al.* [88] studied BMSC plasticity and examined the induced differentiation of BMSC into neuron like cells by using BME or DMSO and BHA. The study confirmed that after induced neuronal differentiation MSC acquired neuron-like morphology and showed an increase in immunolabeling for the neuronal markers NSE and NeuN. However, not only did RT-PCR not confirm these findings, but also other cell types that underwent the same induced differentiation showed similar morphological changes. Careful observations discovered that induced differentiation caused cellular shrinkage of BMSC rather than new neurite growth. In parallel, when BMSC were exposed to different toxic agents that caused cellular shrinkage, the cells displayed a neuron-like morphology. Researchers suggested that morphological and immunocytochemical changes are probably a result of cellular stress followed by cell shrinkage and changes in the cytoskeleton and not a result of rapid neuronal differentiation.

Cellular stress and cell fusion must be eliminated to verify MSC's potential for multi-lineage differentiation.

### CONCLUSIONS

This review has attempted to put in perspective many studies performed on MSC. Despite the questions concerning MSC capability to generate multi-lineage tissues, their therapeutic potential must be our main concern. The means to achieve this purpose should be nurtured and further developed. Nevertheless, the mechanisms of action should be investigated to advance their development and progress. Many experiments, taking possible cell fusion into consideration, showed that fusion could not be responsible for the reported conclusions of new tissue generation [89-91].

Exploring the mechanisms that lie behind therapeutic benefits of MSC transplantation may promote our understanding of the endogenous repair processes and the role of MSC in them. However, even at this stage different clues imply that MSC may be involved in endogenous repair of multiple tissues. As described earlier, many studies conclude that there is a population of MSC in mobilized peripheral blood. Additionally, transplanted MSC were found capable of migration, site-specific differentiation and growth factors secretion, particularly in response to tissue injury and to several types of cytokines. MSC potential participation in physiological repair of different tissues offers the possibility of a whole new strategy of treatment. It may mean that the invasive cell delivery as proposed by stem cells therapy will be minimized, if not avoided, and admission of migratory stimulating molecules and stimulation of physiological homing processes will take its place. To promote this idea, in addition to the identification of MSC potential or actual participation in tissue repair, the means to stimulate the physiological process must be found. Meanwhile, stem cells therapy and its derivatives propose a valuable strategy for tissue repair and regeneration.

Many studies discussed here support MSC plasticity. Dedifferentiation of a more primitive subpopulation of MSC or MSC reprogramming to a more pluripotent cell, may be

associated with their wide differentiation potential. Although the precise potency of MSC is still unresolved, adult stem cells, by definition have a limited differentiation potential compared to ESC. What seems to be a disadvantage may prove to be an important benefit in adult stem cells therapy. Transplantation of pluripotent ESC might be dangerous and may create unwanted teratomas while studies of MSC transplantation, with more limited potential than ESC, did not report on the development of tumors or toxic effects.

Different clinical studies performed with MSC have promising results and report no dangerous outcomes of the treatments. However, safety issues of adult stem cell treatment have not yet been determined. Implanted stem cells might differentiate into unrelated tissue cell type that cannot repair or regenerate the injured tissue, and may actually promote degeneration. Integration of stem cells to the implanted tissue might be necessary for its regeneration and therapeutic effects, even though many therapeutic effects of the transplanted MSC are related to the secretion of repair promoting growth factors that improve endogenous tissue repair and may strengthen the intact tissue. Late-onset toxicity, tumor formation and immune responses, especially after culture in non-autologous culture supplements should be considered and evaluated. All those potential dangers of cell transplantation should not be seen as obstacles but as challenges to overcome. After all, a danger foreseen is half avoided.

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#### ABBREVIATIONS

MSC	= Mesenchymal stem cells
ESC	= Embryonic stem cells
CNS	= Central nervous system
NSC	= Neural stem cells
BM	= Bone marrow
BMSC	= Bone marrow stromal cells
MAPC	= Multipotent adult progenitor cells
DMEM	= Dulbecco's Modified Eagle's Medium
-MEM	= -Modified Eagle's Medium
FBS	= Fetal bovine serum
Gly-A	= Glycophorin-A
NSE	= Neuron-specific enolase
NeuN	= Neuronal nuclear antigen
NF-M	= Neurofilament M
GFAP	= Glial fibrillary acidic protein
RA	= Retinoic acid

EGF	= Epidermal growth factor
BDNF	= Brain-derived neurotrophic factor
FGF	= Fibroblast growth factor
IBMX	= Isobutylmethylxanthine
dbcAMP	= Dibutyryl cyclic AMP
BHA	= Butylated hydroxyanisole
DMSO	= Dimethyl sulfoxide
bFGF	= Basic fibroblast growth factor
SHH	= Sonic hedgehog
EPC	= Endothelial progenitor cells
G-CSF	= Granulocyte-colony stimulating factor
GM-CSF	= Granulocyte macrophage colony stimulating factor
SDF-1	= Stromal derived factor-1
SCF	= Stem cell factor
i.a.	= Intra-artery
i.v.	= Intravenous
i.p.	= Intraperitoneal cavity
GFP	= Green fluorescent protein
BrdU	= Bromodeoxyuridine
TBI	= Traumatic brain injury
NGF	= Nerve growth factor
VEGF	= Vascular endothelial growth factor
VEGFR2	= Vascular endothelial growth factor receptor
MCP-1	= Monocyte chemoattractant protein-1
MIP-1f	= Macrophage inflammatory protein-1f
IL-8	= Interleukin-8
PD	= Parkinson's disease
MPTP	= 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
GDNF	= Glial cell line-derived neurotrophic factor
CSF	= Cerebrospinal fluid
PDGF	= Human platelet derived growth factor-AA
HRG	= Human heregulin-beta1
MLD	= Metachromatic leukodystrophy

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