Gene Therapy (2008) 15, 109–116

www.nature.com/gt

REVIEW

Human mesenchymal stem cells: from basic biology to clinical applications

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Mesenchymal stem cells (MSC) are a group of clonogenic cells present among the bone marrow stroma and capable of multilineage differentiation into mesoderm-type cells such as osteoblasts, adipocytes and chondrocytes. Due to their ease of isolation and their differentiation potential, MSC are being introduced into clinical medicine in variety of applications and through different ways of administration. Here, we discuss approaches for isolation, characterization and directing differentiation of human mesenchymal stem cells (hMSC). An update of the current clinical use of the cells is also provided.
Gene Therapy (2008) **15,** 109–116; doi:10.1038/sj.gt.3303067; published online 8 November 2007

Keywords: *hMSC; stem cell; clinical applications; cell therapy*

Introduction

Human bone marrow-derived mesenchymal stem cells (hMSC) (also known as skeletal stem cells, bone marrow stromal cells or as recently suggested by the International Society for Cytotherapy: multipotent mesenchymal stromal cells[1]) are a group of clonogenic cells present among the bone marrow stroma and capable of multilineage differentiation into mesoderm-type cells such as osteoblast, adipocyte and chondrocyte[2] and possibly, but still controversial, other non-mesoderm type cells, for example, neuronal cells or hepatocytes.[3,4] Moreover, hMSC provide supportive stroma for growth and differentiation of hematopoeitic stem cells (HSC) and hematopoiesis.[5]

The identification and characterization of MSC has been initiated through the pioneering work of Friedenstein in Russia[6] and later Owen and co-workers in UK[7] where culture systems for expanding the cells and for studying their biological characteristics *in vitro* and *in vivo* have been established. Recently, there has been an increased interest in understanding the biology of MSC due to their potential use for therapy of a variety of diseases. The aim of this review is to provide an update related to the biology of hMSC and the challenges facing their use in therapy.

Isolation and characterization of hMSC

hMSC are fusiform, fibroblast-like cells. During their initial growth *in vitro*, they form colonies (termed in analogy with HSC: colony forming unit-fibroblasts [CFU-f]).[7–9] The cells are negative for hematpoietic surface markers: CD34, CD45, CD14 and positive for a variety of markers: Stro-1, CD29, CD 73, CD90, CD105, CD166 and CD44.[1,10,11] Differences exist among the reported studies in the surface marker characteristics that may be explained by variations in culture methods and/or differentiation stage of the cells. However, a number of surface markers has been suggested by a working group within the International Society for Cytotherapy as a minimal criteria for defining the cells as MSC.[1] Traditionally, hMSC have been isolated from low-density mononuclear cell population of bone marrow, based on their selective adherence, compared to hematopoietic cells, to plastic surfaces.[8,9,12,13] One disadvantage of this method is the unavoidable hematopoietic cell contamination and the cellular heterogeneity of cultures with respect of differentiation potential. The concept of cellular 'heterogeneity' of MSC refers to differences in the differentiation potential among single cell clones of MSC. For example, *in vitro* single cell cloning of hMSC has demonstrated that only around 30% of the clonal MSC (that is, CFU-f) are multipotential and thus true MSC.[14] However, there are no current surface markers that can be employed to isolate the multipotent MSC prospectively.

During the recent years, several investigators have tried different methods to enrich the cultures for the multipotent MSC. One approach was to employ monoclonal antibodies in order to isolate a homogenous population of cells with defined phenotype. One of the first antibodies shown to enrich for hMSC is STRO-1 antibody, which identifies an as yet uncharacterized cell surface epitope expressed by hMSC and erythrocytic cells.[15] An enrichment of hMSC has also been tried with combining STRO-1 antibody with CD106 (VCAM-1) or CD146 (MUC18), (STRO-1+/CD106+ or STRO-1+/CD146+).[16] Also, CD271 (low-affinity nerve growth

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Received 8 July 2007; revised 3 October 2007; accepted 8 October 2007; published online 8 November 2007

factor receptor),[17] CD18 (b2 integrin)[18] or the embryonic stem cell marker: SSEA-4[19] have been tried. While these approaches are helpful in isolating MSC from hematopoietic cells, they do not distinguish multipotent MSC from other cells present within the population of MSC and no major differences have been reported between the biological characteristics of the isolated cell populations using these methods.

Due to the lack of specific 'markers' that define the multipotent MSC, these cells are usually defined in functional terms based on *in vitro* and *in vivo* functional assays. MSC are capable for differentiation under appropriate *in vitro* conditions, to mesoderm-type cells, for example, osteoblasts, adipocytes and chondrocytes.[10,20,21] In addition, 'the gold standard' assay for MSC stemness is based on the ability of the cells to form ectopic bone and bone marrow microenvironment supporting hematopoesis upon implantation in an open system (subcutaneous implantation) in immune deficient severe combined immunodeficiency disease mice (Figure 1). This assay has also been employed to demonstrate the ability of the multipotenital MSC cells to exhibit self-renewal and maintenance of 'stemness' capacity during serial implantations.[22]

Other MSC-like populations

Other approaches have been employed and succeeded to isolate cells from the bone marrow that share similarities with MSC and possess additional interesting features. One cell type that has received much attention is the Multipotent Adult Progenitor Cell (MAPC), which has been isolated from CD45$^-$/Glycoprotein A$^-$ depleted bone marrow-derived mononuclear cell fraction, through selective adherence to laminin-coated plates under low serum condition.[23] The most interesting feature of MAPC is their similarity to human embryonic stem cells with respect to their extensive *in vitro* growth and pluripotency.[24] However, it has been difficult to reproduce these results in other laboratories, since MAPC cannot be isolated prospectively and they do not posses so far specific surface markers for their identification. Side population cells isolated from bone marrow based on Hoechest dye exclusion has been isolated from mice and demonstrated to be able to develop to MSC and also hematopoietic stem cells and may thus represent a more primitive stem cell population in the bone marrow.[25] Recently, by using culture conditions resembling the *in vivo* microenvironment of low oxygen tension, D'Ippolito *et al*.[26] have succeeded to isolate a population of MSC named Marrow-Isolated Adult Multilineage Inducible cells. These cells have been reported to have a better growth and a wider differentiation potential compared to MSC cultured by standard methods. While side-by-side comparison of these different cell population is needed, in a preliminary study no major differences could be detected between these different cell populations.[27]

MSC with similar biological characteristics to those derived from bone marrow, have been isolated from other sources including peripheral blood,[28] umbilical cord blood,[29] synovial membrane,[30] deciduos teeth[31] and

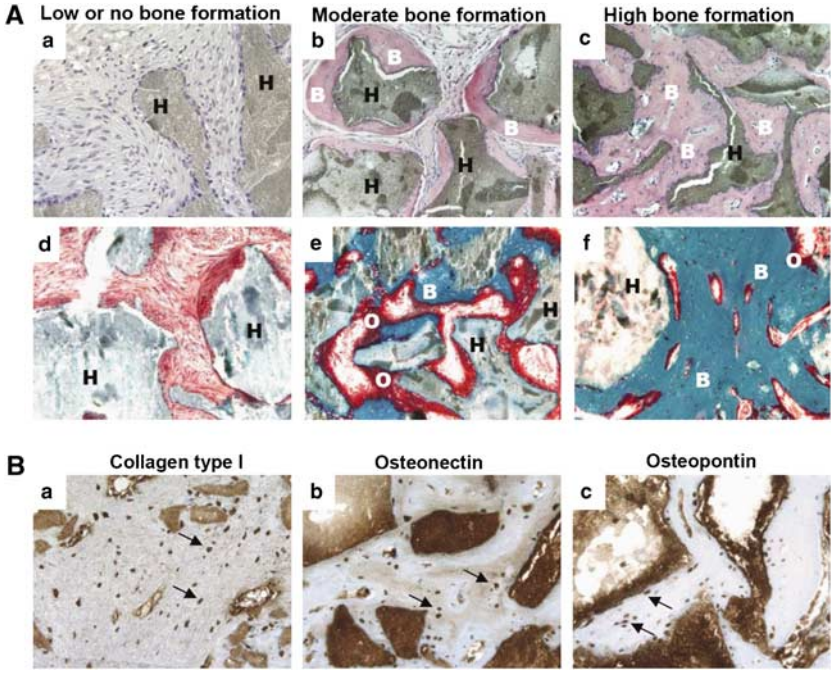

Figure 1 Histological analysis of heterotropic bone formation. (**A**) Histological analysis of heterotropic bone in either decalcified paraffin-embedded implants (a, b and c) or non-decalcified plastic-embedded implants (d, e and f) harvested after 8 weeks of subcutaneous transplantation of hMSC with HA/TCP in immunodeficient mice. The paraffin sections were stained with H&E, and plastic sections were stained with Goldener's Trichrome. Sections that demonstrating the regenerated bone (B), osteoid (O) and HA/TCP. Note that only the Goldener's Trichrome staining could distinguish between osteoid and mineralized bone. Sections show the advantage of using the *in vivo* bone assay to compare the capacity of different hMSC clones to differentiate into bone *in vivo*. Sections were scored as, non-bone forming cells (a and d), cells with moderate bone formation (b and e) and cells with high bone formation (c and f). (Magnification a–f $\times 10$). (**B**) Identifying the origin of heterotropic formed bone in the implants by immunostaining using specific anti-human collagen type I, osteonectin and osteopontin. Arrows show positive stained bone matrix and osteocytes. (Magnification a, b, c $\times 20$).

recently amniotic fluid.[32] These various MSC share some common properties and surface phenotype but differ in their differentiation potential and their gene expression profile in a way that reflect their tissue of origin.[33]

From basic biology to clinical applications

The use of hMSC in clinical applications requires understanding of their biological characteristics and how to employ this information to isolate, expand and differentiate the cells into a particular lineage needed for therapy. Also, novel approaches to administer the cells to the right place in the context of tissue repair and regeneration need to be developed. In the following, we will discuss four areas of translational research that address these points and we will provide an update regarding recent development.

Identification of a homogenous population of hMSC with 'stemness' characteristics

There is a need for identification of surface markers that can be employed prospectively to isolate a homogenous population of multipotent hMSC or their progeny. As seen above, hMSC exhibit a number of surface markers that exhibit a high degree of sensitivity and thus can be employed to obtain a cell population with MSC characteristics. However, these markers lack specificity and thus cannot distinguish between the multipotent MSC and their differentiated progeny or other 'mesenchymal' cells. Our group has tried a global approach to identify new surface markers for hMSC. We performed a quantitative proteomic analysis of isolated membranes of hMSC in undifferentiated and after short-term *in vitro* differentiation using mass spectrometry.[11] Using this method, we have identified 463 unique proteins with extremely high confidence, including typical markers of hMSCs, for example, CD71, CD105, CD166, CD44, Thy1, CD29 and CD63, among 148 integral membranes or membrane-anchored proteins and 159 membrane-associated proteins. A total of 29 integrins and cell adhesion molecules, 20 receptors and 18 Ras-related small GTPases were also identified. Upon differentiation of hMSC to osteoblastic cells, the expression levels of 83 proteins increased by at least twofolds whereas the levels of another 21 decreased by at least twofold.[11] Future studies are needed to validate these potential markers and to employ them in the isolation of multipotent hMSC or their progeny with specific phenotype.

Senescence and limited proliferative potential of hMSC during long-term in vitro *culture*

The clinical use of hMSC requires the availability of a large number of functionally competent cells with stable phenotype. However, *in vitro* expansion of hMSC in long-term culture of hMSC is limited. During long-term *in vitro* culture of hMSC, the cells exhibit reduced proliferation rate and finally enter a state of growth arrest. This *in vitro* phenomenon is termed replicative senescence.[34–36] Replicative senescence is a general phenomenon experienced by all normal somatic diploid cells[37] and it seems to apply for *in vitro* cultured adult stem cells. Our group has studied extensively the *in vitro* 'senescent phenotype' of hMSC. First, we have demonstrated that hMSC exhibit *in vitro* replicative senescence during the current standard *in vitro* culture conditions.[21,34,36] Second, we found that maximal *in vitro* population doubling (PD) of hMSC is dependent on the age of the donor,[21,36] hMSC derived from young donors can be maintained in culture for around 40 PD while hMSC strains derived from elderly donors can be grown in cultures for only 24 PD. Interestingly, we did not detect an donor age-related decrease in the number of the initial clonal hMSC (that is, CFU-F) or in their baseline differentiation potential.[38]

The phenomenon of *in vitro* replicative senescence is caused by several factors including DNA damage, accumulation of abnormal protein or mitochondrial changes.[37,39] However, progressive telomere shortening during continuous *in vitro* growth as the result of absence of telomerase activity in somatic cells, is emerging as a fundamental mechanism.[40,41] Similar to other adult stem cells and in contrast with human embryonic stem cells, we[42] and others[42,43] have demonstrated that cultured hMSC lack telomerase activity because of absence of human telomerase reverse transcriptase (hTERT) gene expression which is the rate limiting factor for telomerase activity in different types of somatic cells. In order to overcome the *in vitro* senescent phenotype, we have overexpressed hTERT in hMSC. This led to restoration of the telomerase activity and elongation of telomere lengths as well as extension of life span of hMSC.[42] In addition, the telomerized hMSC maintained MSC stem cell characteristics *in vitro* and *in vivo*.[10,42] Similar results were obtained in studies that 'telomerized' smooth muscle cells and endothelial cells.[44,45]

Telomerization of the cells is thus an attractive approach for obtaining large number of cells for clinical application.[45] However, in our hMSC–TERT cells, high telomerase activity led to genomic instability and after around 250 PD to cell transformation.[46] It is important to note that this is not an inevitable outcome of hTERT-overexpression. For example, some subpopulations of our telomerized hMSC have been growing in the laboratory for more than 3 years and still maintain full differentiation potential and normal genetic and epigenetic profiles.[46,47] However, from a clinical application point of view, isolation of hMSC with naturally regulated telomerase activity may provide a safer cell type for tissue engineering applications. As mentioned before, some of the isolated hMSC populations seem to express endogenous telomerase activity at least at the culture initiation and at a low levels.[16,26] Alternatively, transient induction of hTERT is another alternative approach. We have recently demonstrated that the lack of telomerase activity in hMSC is due to an epigenetic mechanism and that hTERT expression can be restored transiently by treating the cells with trichostatin A.[48] Thus, it may be possible to identify ways for telomerization of the cells using small chemical molecules. The possibility of obtaining a large number of these cells with maintained genomic stability during long-term culture with these approaches remains to be determined.

Finally, it should be mentioned that other approaches to improve *in vitro* expansion and to extend the *in vitro* life span of hMSC have been reported. Among these treatment of the hMSC with growth factors, for example, FGF2[49,50] or culturing the cells under low oxygen tension[51] or in 3D environment.[52] These physiological

approaches may be useful ways for obtaining large number of cells for clinical applications.

Control of differentiation of hMSC

The multi-potential differentiation capacity of MSC, for example, into bone, cartilage, is the basis for their use in therapy. It is generally accepted that before introducing the cells into patients, they need to be differentiated into a specific lineage since there is a concern that clinical use of undifferentiated stem cells may lead to a situation of uncontrolled proliferation and differentiation resulting in serious complications including tumor formation. However, this hypothesis requires further testing and examination Nevertheless, it is necessary to develop protocols that limit the differentiation potential of hMSC into a particular lineage before their use in therapy. Generally, two approaches have been suggested in the literature to achieve this goal. A genetic approach, where lineage-specific transcription factors have been overexpressed in order to induce lineage-specific MSC differentiation. For examples, for osteoblast differentiation, core-binding factor 1/Runx2,[53] ostrix[54] and lipoprotein-related receptor 5 and its downstream effectors among Wnt signaling molecule[55] can be employed. Peroxisome proliferator-activated receptor-γ 2 (PPARγ2)[56] and Sox9[57] can be employed to induce adipocyte or chondrocyte lineage, respectively. The genetic approach provides important information regarding the biological control of differentiation of hMSC but may not be applicable for generating cells suitable for therapy. Alternatively, a 'micro-environmental' approach can be employed since MSC lineage-specific differentiation depends on the microenvironmental signals received by the cells (the so-called stem cell niche). In this approach, MSC are exposed to different mixtures of growth factors, hormones and extra-cellular matrix components to induce their differentiation. For osteoblast differentiation, several factors have been employed to enhance differentiation, for example, BMPs,[58] Wnt[59] or to inhibit differentiation, for example, Dlk1/Pref-1[60] Noggin.[61]

In order to identify the intracellular signaling pathways that determine the differentiation fate of MSC, we have recently employed a global quantitative proteomic approach to identify phosphotyrosine signaling molecules that are important for MSC differentiation into osteoblasts.[62] We have employed a newly developed quantitative proteomic method called stable isotope labeling in cell culture to compare the signaling pathway initiated by epidermal growth factor (EGF) and that resulted in osteoblast differentiation with that of platelet-derived growth factor (PDGF) that did not. Interestingly, more than 90% of the signaling proteins were utilized by EGF and PDGF, while the phosphatidylinositol 3-kinase pathway was exclusively activated by PDGF implicating it as a possible control point. Indeed, chemical inhibition of PI3K in PDGF-stimulated cells rescued the osteoblast differentiation phenotype[62] (Figure 2). These studies demonstrate the ability of state-of-the-art quantitative proteomic approaches to identify targets for pharmacological intervention in order to control MSC differentiation.

Systemic delivery of MSC

Systemic transplantation of MSC in protocols similar to those utilized in hematpoietic stem cell transplantation

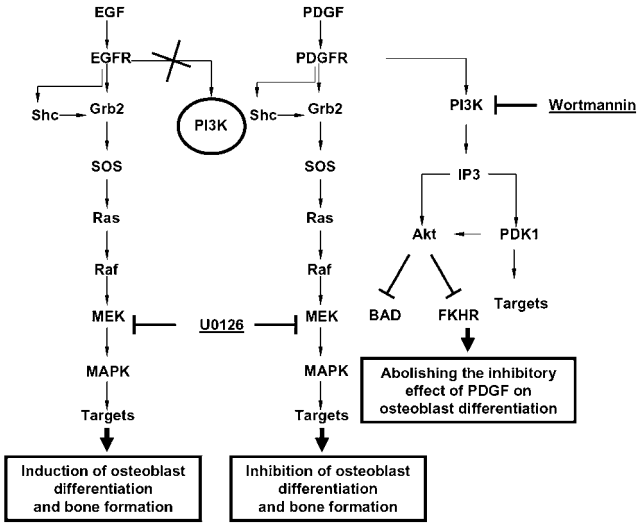

Figure 2 Quantitative signaling proteomic study of osteoblast differentiation using stable isotope labeling by amino acids in cell culture method (SILAC). A quantitative proteomic strategy that metabolically labels the entire proteome, making it distinguishable by MS analysis (SILAC) was used to map the proteomic signaling network initiated by epidermal growth factor (EGF) versus platelet-derived growth factor (PDGF) in hMSC. This quantitative method enable identifying phosphatidylinositol 3-kinase (PI3K) pathway as one of the key regulatory pathway of osteoblast differentiation, where inhibition of PI3K by Wortmannin in PDGF-treated cells leads to enhance osteoblast differentiation and *in vivo* bone formation.

can provide an attractive and clinically relevant method for delivering cells for therapy. However, the challenge is how to improve the engraftment efficiency of MSC to bone marrow and bone[63,64] to allow for biologically relevant effects. MSC can be detected in peripheral blood in different species despite in very low number.[28] The physiological role of the circulating MSC is not known but their presence suggests that that they can home to target tissues and may thus participate in tissue turnover. We have demonstrated that hMSC exhibit a poor ability to cross-capillary barriers when transplanted systemically to non-injured tissues and the majority of cells get entrapped in lung capillaries when administered intravenously.[64] In a recent study,[65] body irradiation has been demonstrated to increase the percentage of cells homing to different organs. Thus, there is a need for further studies to understand and enhance the mechanisms leading to transcapillary migration of MSC and their homing to a specific organ.

Current applications of MSC in therapy

Four areas for potential clinical use of MSC have been explored: local implantation of MSC for localized diseases, systemic transplantation, combining stem cell therapy with gene therapy and use of MSC in tissue engineering protocols.

Local implantation of MSC

Several animal studies have demonstrated the efficacy of using MSC in treatment of bone defects.[66,67] Some clinical case reports have demonstrated the success of locally injected *ex vivo* expanded autologus MSC for treatment

of large bone defects in patients with defective fracture healing.[68] Also, repair of cartilage defects has been tried.[69] Recently, the use of MSC or subset of the cells with vascular endothelial phenotype or a mixture of HSC and MSC stem cell progenitors have been tried in a small number of patients with vascular ischemia secondary to peripheral arterial disease,[70] coronary artery disease[71,72] or non-healed chronic skin wounds.[73] The injected cells were well tolerated and some spectacular results were obtained in some of these trails. However, these initial encouraging results need to be confirmed in randomized clinical trials with adequate number of patients.

Systemic transplantation of MSC

Systemic MSC transplantation has been tried in specific diseases and the results were generally encouraging. Systemic transplantation of allogenic normal bone marrow or purified allogenic MSC has been tried in children with severe osteogensis imperfecta.[74–76] In these studies, engraftment of donor MSC in bone and their ability to differentiate into active osteoblasts has been demonstrated. Also, an increase in the body length and total bone mineral density in the treated children have been demonstrated.[74–76] However, these studies have some limitation especially the number of MSC homed to bone in these patients was small, the patients received other concurrent therapies and the studies did not enroll a control group. Thus, the real contribution of the transplanted MSC to the observed positive clinical response is difficult to determine. Another recent approach for cell therapy of osteogensis imperfecta was performed by in utero transplantation of allogenic fetal MSC into a female fetus with multiple intrauterine fractures at the 32nd week of gestation.[77] Interestingly, engraftment of donor cells and increased bone mineralization have been observed in the bone of the patient at 9 months of age.[77] Case reports of well tolerated allogenic MSC transplantation and some clinical improvement have been reported in patients with Hurler's syndrome and severe idiopathic aplastic anemia.[78,79] Furthermore, combining MSC transplantation with HSC transplantation was tried to increase the homing ability of HSC to bone marrow.[80] Also, a promising area of clinical application is the use of systemic transplantation of MSC in treatment of graft-versus-host-disease during allogenic HSC transplantation.[81,82] The rationale behind the use of MSC in this context is the increasing evidence that MSC exerts immune-regulatory effects.[81,82]

Finally, the field of systemic MSC transplantation is encouraged by the fact that MSC are hypoimmunogenic and thus allowing MSC transplantation between HLA-incompatible individuals.[83]

Combining stem cell therapy with gene therapy

The use of gene-modified stem cells in the context of gene therapy is an attractive option due to theoretical advantage of stem cells compared to somatic cells with respect to higher proliferative capacity and long-term survival. Genetically modified MSC can deliver genes or proteins into organs or tissues with specific need for gene therapy. Some these approaches have been tried in animal models. MSC cells expressing ectopic BMP-2 have been used successfully for repair of articular cartilage and for bone regeneration in animal models.[84,85] In a model for hemophilia treatment, hMSC transduced with a retroviral vector encoding a human B-domain deleted FVIII (hFVIIIdeltaB) cDNA exhibited long-term engraftment, in absence of myeloablative conditioning as well as persistent expression of the therapeutic levels of FVIII in the plasma of recipient mice.[86] Moreover, MSC have been demonstrated to be able to express exogenous proteins (for example, IL-3) for extended period of time and to maintain this ability after transplantation *in vitro*.[87] Therapy based on these genetic modified cells is thus possible.

Tissue engineering

Tissue engineering may provide alternative ways for obtaining tissues and organs needed for transplantation due to lack of sufficient number of organ donors and limitations attributable to immunological rejection and mismatch of physical dimensions. Tissue engineering may allow obtaining patients own cells, seeding them on biodegradable scaffolds that allow formation of a particular tissue.[88] These tissues can be used to repair tissue defects due to disease or trauma. Also, tissue engineering may also allow *ex vivo* engineering of tissue by the means of 3D bio-scaffolds seeded with mature cell or stem cells and cultivated in bioreactors that lead to the formation of tissues or organs, for example, liver, hearts, cartilage or kidneys.[88] MSC are candidates for use in tissue engineering protocols because of the relative ease for establishing the cells *in vitro* and their differentiation potential.[89] Several scaffolds are currently available and may be classified as either: (a) biologically derived polymers isolated from extracellular matrix, plants and seaweed, for example, collagen type I or fibronectin, alginate from brown algae or (b) synthetic, for example, hydroxyapatite, tricalcium phosphate ceramics, polylactide and polyglycolide and a combination of these in the form of poly DL-lactic-co-glycolic acid.[88,89] There exists several animal experiments showing the success of using this approach, for example, for treatment of large bone defects in animal models[90] and for use in tissue reconstruction.[91] It is expected that transplantation of tissues based on these methods to humans will be achieved in the coming years.

Conclusion

Studying the biology of MSC and introducing these cells into the clinic through cell-based therapy protocols is a very exciting area of basic and clinical experimental research. It is expected that MSC with their interesting biological characteristics will provide new approaches to treatment of chronic diseases. In addition, studying their biology will provide insight into basic biological processes of control of cell proliferation, differentiation and tissue turnover. It is hoped that this information will benefit the whole field of regenerative medicine.

Acknowledgements

The study was supported by grants from the Danish Medical Research Council, the Novo Nordisk Foundation, the Vellux Foundation and Danish Center for Stem Cell Research.

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