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Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases

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Abstract

The intriguing biology of stem cells and their vast clinical potential is emerging rapidly for gene therapy. Bone marrow stem cells, including the pluripotent haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and possibly the multipotent adherent progenitor cells (MAPCs), are being considered as potential targets for cell and gene therapy-based approaches against a variety of different diseases. The MSCs from bone marrow are a promising target population as they are capable of differentiating along multiple lineagesn and, at least *in vitro*, have significant expansion capability. The apparently high self-renewal potential makes them strong candidates for delivering genes and restoring organ systems function. However, the high proliferative potential of MSCs, now presumed to be self-renewal, may be more apparent than real. Although expanded MSCs have great proliferation and differentiation potential *in vitro*, there are limitations with the biology of these cells *in vivo*. So far, expanded MSCs have failed to induce durable therapeutic effects expected from a true self-renewing stem cell population. The loss of *in vivo* self-renewal may be due to the extensive expansion of MSCs in existing *in vitro* expansion systems, suggesting that the original stem cell population and/or properties may no longer exist. Rather, the expanded population may indeed be heterogeneous and represents several generations of different types of mesenchymal cell progeny that have retained a limited proliferation potential and responsiveness for terminal differentiation and maturation along mesenchymal and non-mesenchymal lineages. Novel technology that allows MSCs to maintain their stem cell function *in vivo* is critical for distinguishing the elusive stem cell from its progenitor cell populations. The ultimate dream is to use MSCs in various forms of cellular therapies, as well as genetic tools that can be used to better understand the mechanisms leading to repair and regeneration of damaged or diseased tissues and organs.

Keywords

gene therapy; mesenchymal stem cells; vector

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1. The advent of stem cells in gene therapy

Over the past two decades, the ability to transfer genes into stem cells has raised hopes towards the feasibility of using gene therapy-based approaches to provide long-term therapeutic impacts [1,2]. Numerous studies have deepened our understanding of the behaviour of individual stem cells in different tissue microenvironments. In addition, the development of better assays for stem cells and improvements in vector biology have increased gene transfer efficiencies into both haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). It is envisaged that a thorough evaluation of human gene therapy protocols will lead to a better understanding of the potential of stem cells in gene therapy approaches directed towards both inherent and acquired diseases. Recently, the excitement concerning HSCs and MSCs has been dampened somewhat by the interest surrounding embryonic stem cells (ESCs), primarily due to the fact that HSCs and MSCs are viewed to be limited in terms of their potential to differentiate into specific cell types, whereas ESCs can potentially differentiate into any cell type. The possible uses of ESCs to treat human disease, however, are highly controversial both for moral reasons and based on clinical findings indicating that the use of ESCs in long-term gene therapy protocols may carry risks due to the neoplastic potential of these highly proliferative cells.

The unique biology of stem cells and their vast clinical potential is emerging rapidly [3]. The bone marrow (BM) is often used as a provider of stem cells for gene therapy approaches. The BM is composed of both non-adherent haematopoietic and adherent stromal cell compartments. Both the HSCs and the MSCs can self-renew by proliferation and maintain their stem cell phenotype. The HSCs give rise to all different blood cell lineages, such as the myeloid and lymphoid cell lineages, and MSCs give rise to the stromal cells, which belong to the osteogenic, chondrogenic, adipogenic, myogenic and fibroblastic lineages. A more primitive adherent stem cell has recently been identified. This multipotent adult progenitor cell (MAPC) population can differentiate into MSCs, endothelial, epithelial and even haematopoietic cells [4]. BM stem cells, including the pluripotent HSCs, MSCs and possibly the primitive MAPCs, are being considered as potential targets for cell and gene therapy-based approaches against a variety of different diseases [5-7]. Although the use of stem cells may not overcome the usefulness of traditional medicines, gene therapy strategies involving stem cells in conjunction with the available drug regimens may help in better treatment options of otherwise incurable diseases.

2. The potential of mesenchymal stem cells in stem cell gene therapy

In the past few years, the use of MSCs in both cell-based and gene-based therapies has gained momentum [7-9]. MSCs from BM are capable of differentiating along multiple lineages and, at least *in vitro*, have significant expansion capability. There is mounting evidence that these cells will ultimately be useful as vehicles for cell and gene therapies, especially in the field of tissue engineering. The ultimate goal is to use MSCs in various forms of therapy, as well as tools to understand the mechanisms leading to repair and regeneration of damaged or diseased tissues and organs. This approach has provided a lot of promise in the treatment of bone disorders as well as vascular diseases. The long lifespan and homing ability of MSCs are attractive assets in the context of gene therapy strategies directed against infectious diseases and metastatic tumours. The use of MSCs in different therapeutic strategies either as immunosuppressive agents or as vehicles to express therapeutic proteins acting against autoimmune processes have been discussed by Jorgensen *et al.* [9]. There is emerging evidence that MSCs deploy a very powerful array of mechanisms that allow their escape from host allogeneic responses. These mechanisms include limited expression of alloantigen by MSCs and cell contact-dependent and -independent mechanisms. Their phenotype characteristics indicate that they have MHC class I antigens, but lack MHC class II, and costimulatory molecules CD40, CD80 and CD86; indicating that MSC class I antigens may stimulate alloreactive T cells, but MSCs can not engage in secondary signalling as they lack

costimulatory molecules. More importantly, MSCs appear to modulate host dendritic cell and T cell function, promoting induction of suppressor or regulatory T cells. These effects are complemented by the induction of divisional arrest anergy in T cells and by the production of soluble immunomodulatory factors, including interleukin (IL)-10), transforming growth factor-beta, prostaglandin E2, and hepatocyte growth factor. These mechanisms allow MSCs to inhibit the production of cytotoxic lymphocytes and natural killer cells *in vitro* and prolong skin allograft survival in immunocompetent outbread baboons. In addition, MSCs express the enzyme indoleamine 2,3-dioxygenase, which creates a tryptophan-depleted milieu that promotes immunosuppression. These observations show a striking similarity to emerging data on the maternal acceptance of the fetal allograft [10]. Although the limited *in vitro* and *in vivo* observations suggest that MSCs may be potentially used to induce tolerance into allogeneic or xenogeneic hosts, more studies are needed to fully understand host immune responses to cultured MSCs as well as their immunomodulatory mechanisms for facilitating unrelated HSC transplantation, minimising graft-versus-host disease and preventing rejection for organ transplantation.

Genetically manipulated MSCs may have direct applications to impact diseases in a variety of cell types in elaborate microenvironments and in different tissues *in situ*. The ability to genetically modify MSCs provides a means for durable expression of therapeutic genes for the lifetime of the patient for a wide range of diseases. MSCs can be engineered to secrete a variety of different proteins *in vitro* and *in vivo* that could potentially treat a variety of serum protein deficiencies and other genetic or acquired diseases, including bone, cartilage and BM disorders, or even cancer. Improvements in gene delivery into HSCs have provided clues towards crucial improvements required to enhance therapeutic efficacy of MSCs for a variety of different diseases. A better understanding of the molecular mechanism directing the differentiation of MSCs will eventually allow to properly manipulate MSCs both *ex vivo* and *in vivo* to allow the regeneration of complex tissues and organs.

3. Transgene delivery into mesenchymal stem cells

Various approaches are available to introduce transgenes into MSCs. Viral vectors permit efficient transgene delivery. However, safety concerns associated with viral transduction have prompted a search for alternative non-viral gene delivery methods.

3.1 Transgene delivery into mesenchymal stem cells using viral vectors

3.1.1 Vectors based on oncogenic retroviruses—The ability of MSCs to self-renew at a high proliferation rate led to the prediction early on that they would be ideal targets for retrovirus-mediated transgene delivery strategies [11]. A variety of studies using vectors based on oncogenic retroviruses have attempted to transduce MSCs, but there have been problems due to a number of issues. A major limitation of transduction approaches involving oncogenic retroviral vectors such as Moloney murine leukaemia virus (MoMLV) is a general lack of long-term transgene expression [12,13], possibly due to the inactivation of the retroviral long terminal repeat vectors based on murine stem cell virus appear to be less prone to transcriptional silencing of viral gene expression and, thus, appear to be more promising. Marx *et al.* [14] have shown that both genes of a bicistronic vector based on murine stem cell virus were expressed for at least 6 months in human MSCs *in vitro*. In a related study, transgene expression from murine stem cell virus-based vectors *in vivo* lasted for up to 12 weeks in human MSCs, adhered to ceramic cubes and implanted into severe combined immunodeficient (SCID) mice [15]. However, transduction of MSCs with MoMLV and murine stem cell virus-based vectors were shown to be inefficient, as they required drug selection to enrich transduced cells [13,15], multiple rounds of transduction for several days [14,16,17], or highly concentrated vector stocks [18]. In addition, efficient transduction of human MSCs by amphotropic MoMLV and murine stem cell virus pseudotypes was found to be limited by the expression levels of the

amphotropic viral receptor. The amphotropic receptor is a phosphate transporter whose expression is increased in the absence of phosphate. Chuah *et al.* [16] used a phosphate starvation procedure to increase transduction of MSCs by amphotropic MoMLV-based vectors.

3.1.2 Lentivirus-based vectors—Recent results from several labs have indicated that HIV-1-based vectors are very efficient at delivering and expressing transgenes into MSCs [19-24]. A single round of transduction using unconcentrated HIV-1-based lentiviral vectors led to the efficient transduction of human MSCs and sustained transgene expression for up to at least 5 months [19]. An advantage of lentiviral vectors over vectors based on oncogenic retroviruses is that they are capable of transducing non-dividing cells [25,26]. This is important given the fact that a relatively large subset (20%) of mesenchymal progenitor cells (MPCs) has been described to be quiescent [27]. Results reported by Zhang *et al.* [22] have shown that whereas transduction efficiencies with lentivirus particles pseudotyped with the vesicular stomatitis virus (VSV)-G glycoprotein were high, RD114 pseudotypes bearing the feline endogenous virus RD114 glycoprotein revealed transduction efficiencies that were 1 - 2 orders of magnitude below those observed with VSV-G pseudotypes. However, chimeric RD114 glycoproteins, with the transmembrane and extracellular domains fused to the cytoplasmic domain derived from the amphotropic MoMLV 4070A Env glycoprotein, revealed ~ 15-fold higher titres relative to the unmodified RD114 glycoprotein. The transduction efficiencies in human MSCs of HIV-1-based vectors pseudotyped with the chimeric RD114 glycoprotein were similar to those obtained with HIV-1 vectors pseudotyped with VSV-G. The results reported by Zhang *et al.* also indicated that RD114 pseudotypes were less toxic than VSV-G pseudotypes in human MSC progenitor assays [22].

Lee *et al.* [21] have used self-inactivating HIV-1-based lentiviral vectors in the context of fetal rhesus monkey BM-derived MSCs. Flow cytometric analyses indicated an 8- to 10-fold greater quantity of green fluorescent protein (GFP)-expressing rhesus MSCs when cells were transduced with vectors bearing the cytomegalovirus immediate-early or translation elongation factor-1α promoters compared to the phosphoglycerate kinase promoter. Transduced rhesus MSCs differentiated towards an osteogenic lineage comparable to untransduced MSCs. In agreement with the reports published by Zhang *et al.* [19], these findings suggest that HIV-1-derived lentiviral vectors can efficiently transduce rhesus MSCs *in vitro* without inhibiting their differentiation potential.

Anjos-Alfonso *et al.* [28] described a method of purifying murine MSCs from BM and for efficiently transducing them using lentiviral vectors. Lentivirus-transduced mouse MSCs retained their *in vitro* ability to differentiate into adipocytes, osteocytes and chondrocytes as well as into myocyte- and astrocyte-like cells. Transduced MSCs were delivered systemically into minimally injured syngeneic mice. Tracking and tissue-specific differentiation were determined by polymerase chain reaction (PCR) and immunohistochemistry, respectively. Donor-derived hepatocytes, lung epithelial cells, myofibroblasts, myofibres and renal tubular cells were detected in some of the recipient mice. These data indicate that even in the absence of substantial injury, phenotypically defined murine MSCs can acquire tissue-specific morphology and antigen expression, and thus contribute to different tissue cell types *in vivo*.

3.1.3 Adenoviral vectors—Transgene delivery by unmodified adenoviral (Ad) vectors appears to be inefficient as far as MSCs are concerned. Conget and Minguell [29] have used Ad vectors to deliver reporter genes into *ex vivo* expanded MPCs. Only ~ 19% of the cells expressed the transgene, possibly due to the absence of the corresponding Coxsackie adenovirus receptor receptor on such cells [30]. To overcome this problem, Olmsted-Davis *et al.* [31] have designed chimeric Ad vectors to improve transgene delivery into MSCs. The vectors used consisted either of a standard Ad Type 5 (Ad5) vector or a chimeric Ad5 vector that contained an Ad Type 35 fibre (Ad5F35). Human MSCs transduced with Ad5F35 vectors

displayed higher levels of transgene expression than those transduced with unmodified Ad5 vectors. In a related attempt to increase the efficiency of gene transfer into rat MSCs using Ad vectors, Tsuda *et al.* [32] used a fibre-modified Ad5 vector (Ad/RGD) containing an RGD-containing peptide in the HI loop of the fibre knob domain. Transduction efficiencies into MSCs with the Ad/RGD vector were increased 12-fold compared with a vector containing an unmodified HI loop.

3.1.4 Vectors based on adeno-associated virus—Vectors based on adeno-associated virus (AAV) have found limited applications in MSCs so far due to low transduction efficiencies. To overcome these shortcomings, Ito *et al.* [33] used an ultraviolet (UV) light-activated transduction system to improve the delivery of AAV vectors into human MSCs. This procedure involving UV irradiation had no effect on either the chondrogenic or osteogenic potential of MSCs. A recent report by Kumar *et al.* described optimised conditions for AAV-mediated gene transfer into murine MSCs [34].

3.1.5 Alternative viral vector systems—Although transduction efficiencies of up to 95% were observed with herpes virus saimirii (HVS)-based vectors [35], the generation of safe replication-deficient HVS vector stocks remains a major issue. This problem may limit future clinical applications with MSCs involving HVS vectors.

3.2 Transgene delivery into mesenchymal stem cells using non-viral methods

To bypass safety concerns associated with viral vectors, alternative, non-viral based methods for transgene delivery were established for MSCs. Traditional transfection methods have shown little success in delivering plasmid DNA into primary MSCs, usually resulting in low transfection efficiencies and high cell mortality. Song *et al.* recently described the development of a novel, noninvasive transgene delivery protocol, based on the principle of electric field-induced molecular vibration [36]. This method enabled foreign DNA molecules to penetrate the plasma membrane and to enter the cytoplasm of MPCs at high efficiency and with low cell mortality. This promising procedure did not interfere with the normal cellular differentiation activities of human and chick mesenchymal progenitors.

Peister *et al.* [37] developed improved conditions for stable transfection of human MSCs by electroporation. Following selection using G418, the transfected MSCs could be expanded 300-fold in 14 days and 98% of the progeny cells expressed the transgene. Stable transfection of plasmid DNA into rat MSCs by electroporation was also successful. The transfected MSCs retained their capacity to differentiate into both adipocytes and osteoblasts. Thus, MSCs were stably transfected with plasmid DNA and retained their differentiation capacity after expansion.

In a recent report, Hoelters *et al.* [38] described liposome-based transfection methods to introduce transgenes and small interfering RNAs (siRNAs) into human MSCs. Transfected MSC maintained their proliferation capacity paired with the ability to differentiate into different mesodermal lineages (bone, cartilage and fat) without loss of transgene expression.

Vanderbyl *et al.* [39] used mammalian artificial chromosomes (ACEs) for stable transgene delivery and expression in human MSCs. Fluorescent *in situ* hybridisation and fluorescent microscopy demonstrated that the ACEs were stably maintained as single chromosomes that expressed the transgene in differentiated cultures. These findings demonstrate the potential usefulness of ACEs for *ex vivo* gene therapy of MSCs.

4. Genetic modification of mesenchymal stem cells to express recombinant proteins

4.1 Mesenchymal stem cells as platforms for recombinant protein production *in vitro*

To assess the capacity of MSCs to produce heterologous proteins, many different transgenes were expressed in MSCs *in vitro*. The proteins included the *Escherichia coli* β-galactosidase [15,40], GFP [14,18,19] and red fluorescent protein (DsRed) [19], as well as many therapeutic proteins, including coagulation factors VIII [12,16,17] and IX [41-43], IL-3 [15,44,45] and IL-7 [46], human growth hormone [41], human erythropoietin (hEPO) [47] and murine erythropoietin (mEPO) [48], arylsulfatase A [49,50], tyrosine hydroxylase GTP cyclohydrolase I [13,51], α-L-iduronidase [52], β-hexosaminidase A [53] and bone morphogenetic protein (BMP) [54]. It remains to be determined how MSCs perform relative to other mammalian expression systems, such as Chinese hamster ovary cells, in terms of transgene expression levels.

To isolate regulators of osteogenesis, metastasis and angiogenesis, Michiels *et al.* constructed and validated an individually arrayed, replication-defective adenoviral library harbouring human placental cDNAs, termed PhenoSelect library [55]. The arrayed PhenoSelect library was screened in cellular assays involving MSCs. This resulted in the identification of known proteins, as well as novel proteins that were not known to play roles in these pathways. These results indicate that MSCs provide a potent screening system to unravel the functions of proteins.

4.2 Marking of mesenchymal stem cells for *in vivo* tracking

A number of reports have dealt with the *in vivo* distribution of MSCs marked with reporter genes. Brouard *et al.* [56] used MoMLV-based retroviral vectors encoding the mouse CD2 antigen to mark STRO-1^+ cells selected from adult and fetal BM. Gene-modified stromal cells were injected intravenously into non-obese diabetic (NOD)/SCID mice engrafted with pieces of human fetal haematopoietic bone. Using nested PCR, transgenic human cells were detected both in the marrow of human bone grafts and in the BM, liver and spleen of host mice 7 weeks after grafting. These data indicate that BM stromal pogenitor cells can home to haematopoietic tissues on engraftment through the bloodstream of non-conditioned hosts.

The capacity of mouse MSCs to contribute to different cell types *in vivo* is unclear. To investigate this question, Anjos-Afonso *et al.* [28] described a method to purify murine MSCs from BM to efficiently transduce them using a lentiviral vector expressing the GFP reporter gene. Lentivirus-transduced mouse MSCs retained their *in vitro* ability to differentiate into adipocytes, osteocytes and chondrocytes as well as myocyte- and astrocyte-like cells. GFP-positive MSCs were then delivered systemically into minimally injured syngeneic mice. Tracking and tissue-specific differentiation of MSCs were determined by PCR and immunohistochemistry, respectively. Donor-derived hepatocytes, lung epithelial cells, myofibroblasts, myofibres and renal tubular cells were found in some of the recipient mice. These data show that even in the absence of substantial injury, phenotypically defined MSCs can acquire tissue-specific morphology and antigen expression, and thus contribute to different tissue cell types *in vivo*.

Devine *et al.* carried out *in vivo* tracking experiments involving MSCs in baboons [57]. They infused *ex vivo*-expanded MSCs transduced with a retroviral construct encoding GFP into 3 adult baboons following lethal total body irradiation and haematopoietic support or without any prior conditioning. To study the long-term fate of these MSCs, necropsies were performed between 9 and 21 months following MSC infusion, and an average of 16 distinct tissues were recovered from each recipient and evaluated for the presence of the GFP transgene in purified

genomic DNA using a sensitive real-time PCR approach. Two baboons received autologous MSCs and one received allogeneic MSCs expressing GFP. Both allogeneic and autologous MSCs appeared to distribute in a similar manner. Gastrointestinal tissues harboured high concentrations of transgene per microgram of DNA. Additional tissues, including kidney, lung, liver, thymus and skin, were also found to contain relatively high amounts of DNA equivalents. Estimated levels of engraftment in these tissues were in the range of 0.1 - 2.7%. The non-conditioned recipient appeared to have less abundant engraftment. These data suggest that MSCs initially distribute broadly following systemic infusion and that they may participate later on in ongoing cellular turnover and replacement in a wide variety of tissues.

To track the successful delivery, homing and localisation of MSCs to the site of myocardial injury, Hill *et al.* [58] used MSCs labelled with fluorophore particles (IFPs) to provide magnetic resonance imaging (MRI) contrast *in vivo*. The authors concluded that IFP labelling of MSCs imparts useful MRI contrast, enabling ready detection in the beating heart on a conventional cardiac magnetic resonance (MR) scanner after transplantation into normal and infarcted myocardium. The dual-labelled MSCs could be identified at locations corresponding to injection sites, both *ex vivo* using fluorescence microscopy and *in vivo* using susceptibility contrast on MRI. This technology may permit effective *in vivo* studies of stem cell retention, engraftment and migration. Dick *et al.* have developed a technique that used MR fluoroscopy to guide intramyocardial MSC injection to desirable targets, such as the border between infarcted and normal tissue [59]. MR fluoroscopy allowed visualisation of catheter navigation, myocardial function, infarct borders and labelled cells after injection.

4.3 Mesenchymal stem cells as platforms for recombinant protein production *in vivo* to treat acquired and inherited disorders

4.3.1 Gene-modified mesenchymal stem cells to treat neurological disorders— Gene-modified MSCs provide attractive platforms for the sstained production of therapeutic proteins *in vivo*. Progress along those lines has been made in rodent models of neurodegenerative disorders, such as Parkinson's disease [13,60], and lysosomal storage disorders, including Tay-Sachs disease [53], Niemann-Pick disease types A and B [61,62], and mucopolysaccharidosis Type VII [63].

MSCs were recently also reported to ameliorate functional deficits after stroke induction in rats. Kurozumi *et al.* [64] engineered MSCs to express brain-derived neurotrophic factor (BDNF) to promote functional recovery and to reduce infarct size in the rat middle cerebral artery occlusion model. MRI analysis revealed that the rats in the MSC-BDNF group exhibited more significant recovery from ischaemia after 7 and 14 days compared with unmodified MSCs. These data suggest that MSCs expressing BDNF may be useful in the treatment of cerebral ischaemia and may represent a new strategy for the treatment of stroke.

4.3.2 Gene-modified mesenchymal stem cells to treat blood disorders—Other inherited disorders, including haemophilia A [12,65] and haemophilia B [66], have also been targeted using MSC-based cell therapy approaches *in vivo*. Bartholomew *et al.* [47] used baboon MSCs to express hEPO *in vivo*. In parallel experiments, transduced MSCs were injected intramuscularly in NOD/SCID mice. In a separate experiment, transduced MSCs were loaded into immunoisolatory devices that were surgically implanted into either autologous or allogeneic baboon recipients. hEPO was detected in the serum of NOD/SCID mice for up to 28 days and in the serum of five baboons for up to 137 days. NOD/SCID mice experienced sharp rises in haematocrit after intramuscular injection of hEPO-transduced MSCs. The baboons that expressed hEPO for 137 days displayed a statistically significant rise in its haematocrit. In a related study, Eliopoulos *et al.* [48] determined if gene-modified mouse MSCs sequestered within a clinically approved, bovine Type I collagen-based viscous bulking

material could serve as a retrievable implant for systemic delivery of mEPO. To test this approach, they embedded mEPO-secreting MSCs in viscous collagen and determined the pharmacological effect following implantation in normal mice. To do this, primary MSCs from C57Bl/6 mice were retrovirally transduced to express mEPO and cells of a clonal population secreting mEPO were implanted subcutaneously in normal C57Bl/6 mice with and without viscous collagen present. Without matrix support, haematocrit values rose to > 70% for < 25 days and returned to baseline by 60 days. However, in mice implanted with viscous collagen-embedded mMSCs, the haematocrit rose to > 70% for up to 203 days post implantation. Surgical removal of the viscous collagen organoid 24 days after implantation led to a reduction of haematocrit to baseline levels within 14 days. This investigation demonstrates that MSCs embedded in a human-compatible viscous collagen matrix offers a potent, durable and reversible approach for delivering therapeutic proteins.

Allay *et al.* [15] investigated human IL-3 (hIL-3) expression in human MSCs transduced with a myeloproliferative sarcoma virus-based oncogenic retroviral vector encoding hIL-3. Transduced cells implanted into SCID mice formed bone and secreted detectable levels of hIL-3 into the systemic circulation for at least 12 weeks. In a related study, Lee *et al.* analysed the stability of transgene expression in human MSCs after differentiation *in vitro* and *in vivo* [44]. Long-term *in vitro* and *in vivo* expression (> 6 months) of hIL-3 was observed in human MSCs following gene transfer involving oncogenic retroviral vectors. Transduced MSCs were able to differentiate into osteogenic, adipogenic and chondrogenic lineages, and maintained transgene expression after differentiation. Parallel studies were performed *in vivo* using NOD/SCID mice. Human MSCs expressing hIL-3 were cultured on several matrices and then delivered by subcutaneous, intravenous, and intraperitoneal routes. Sampling of peripheral blood demonstrated that systemic hIL-3 expression was maintained in the range of 100 - 800 pg/ml over a period of 3 months. These results illustrate the capacity of human MSCs for sustained expression of therapeutic proteins and demonstrate their potential clinical usefulness as cellular vehicles for systemic gene delivery.

4.3.3 Gene-modified mesenchymal stem cells to treat vascular diseases—

Cardiovascular disease (CVD) is the leading cause of death in the US, and the use of stem cells in the treatment of the various anomalies that precipitate the CVD has enormous potential. There is growing evidence that MSCs can be used for regenerating the myocardium and blood vessels [67]. When expanded *ex vivo*, they expressed markers for myocardial and endothelial cells [68-70]. Fukuda *et al*. isolated a murine cardiomyogenic cell line (CMG cell) from murine BM MSCs [71]. The cells changed morphology after exposure to 5-azacytidine and started beating within 2 weeks. Upon molecular characterisation, these cells were found to express alpha 1A, alpha 1B, alpha 1D, beta 1 and beta 2 adrenergic, and M1 and M2 muscarinic receptors, and they also responded to alpha and beta adrenergic agonists and antagonists. These studies open up new avenues in the use of MSCs in the treatment of cardiovascular disease. The findings indicate that cell transplantation therapy for patients with heart failure may be possible in the future by using regenerated cardiomyocytes derived from autologous BM cells [72,73]. In earlier studies, Cheng *et al.* used human cord blood MPCs and MSCs to regenerate cardiomyocytes [74]. These cells were placed in a medium containing low serum concentrations, and were allowed to adhere and then expanded in the medium supplemented with 5-azacytidine. Staining for cardiogenic-specific contractile protein troponin T was performed to identify cardiomyocyte-like cells. After cardiogenic induction, 70% of cord blood-derived mesenchymal progenitor cells differentiated into cardiomyocyte-like cells. In a similar study, Xu *et al.* treated human MSCs with 5-azacytidine to investigate their differentiation into cardiomyocytes. The myogenic cells that differentiated from MSCs were positive for beta-myosin heavy chain, desmin and alpha-cardiac actin. These cells also responded to stimulation with K(+) (5.0 mM) by increasing intracellular calcium [75]. The results of these studies indicate that 5-azacytidine can induce human MSCs to differentiate *in*

vitro into cells with characteristics commonly attributed to cardiomyocytes. In another study using a differentiation medium containing insulin, dexamethasone and ascorbic acid, human MSCs were differentiated into cardiomyocyte-like cells (CLCs) [68]. Differentiated CLCs expressed cardiac troponin I, sarcomeric tropomyosin, and cardiac titin. A theory that the myocardial microenvironment plays a critical role in determining the fate of MSCs was put to test in a study performed by Rangappa *et al.* [76]. In this study, human MSCs were cultured in the presence of human cardiomyocytes ('co-culture') or in the presence of conditioned media obtained from separate cultures of human cardiomyocytes ('conditioned media'). The results of this study showed that human MSCs co-cultured with cardiomyocytes differentiated into cardiomyocytes, whereas human MSCs exposed to conditioned media did not. Differentiated human MSCs from the co-culture experiments expressed myosin heavy chain, beta-actin and troponin T. This study indicates that in addition to soluble signalling molecules, direct cell-to-cell contact may be essential in relaying the external cues of the microenvironment controlling the differentiation of adult stem cells to cardiomyocytes [76]. Toma *et al.* in an earlier study showed that human MSCs when injected directly into the myocardium of mice can differentiate into cardiomyocytes expressing the cardiac markers, desmin, β-myosin heavy chain, α-actinin, cardiac troponin T and phospholamban at levels comparable to those of the host cardiomyocytes [77]. In a rat model of acute myocardial infarction, Nagaya *et al*. showed that rats that were transplanted intravenously with MSCs had improved cardiac function through enhancement of angiogenesis and myogenesis in the ischaemic myocardium compared to the control group [78]. These MSCs were isolated from BM aspirates of isogenic adult rats and expanded *ex vivo*. The engrafted MSCs were positive for cardiac markers such as desmin, cardiac troponin T and connexin 43. Some of the MSCs were positive for the endothelial cell marker von Willebrand factor [79]. This shows that systemically delivered MSCs have a therapeutic potential in treating myocardial ischaemia.

Electronic cardiac pacemakers have emerged as an important therapeutic tool in the treatment of patients with high-degree heart block and sino-atrial node dysfunction. The sino-atrial node is the primary biological pacemaker in the heart. Electronic pacemakers mimic the function of the sino-atrial node. It would be therapeutically advantageous if the electronic pacemaker could be replaced by a biological one. Therefore, it is important to test the potential of MSCs to differentiate into cells that have functional characteristics of a sino-atrial cell. When human MSCs were transfected with a cardiac pacemaker gene, mHCN2, and injected subepicardially in the canine left ventricular wall *in situ*, they expressed functional HCN2 channels mimicking overexpression of HCN2 genes in cardiac myocytes [80].

Successful vascularisation of the myocardium and of other engineered tissues such as artificial bone and cartilage is extremely important for the survival of the tissue. Koike *et al.* [79] demonstrated that when MSCs are co-cultured with endothelial cells, they form long lasting and stable blood vessels. In this study, the investigators co-cultured human umbilical vein endothelial cells (HUVECs) with MSCs, which were then implanted in mice. HUVECs formed long, interconnected tubes with many branches that subsequently connected to the mouse's circulatory system and became perfused. In contrast, constructs prepared from HUVECs alone showed minimal perfusion. To confirm their incorporation into the vessel wall, MSCs were fluorescently labelled. Oswald *et al.* [81] used 2% fetal calf serum and 50 ng/ml vascular endothelial growth factor (VEGF) as supplements to differentiate MSCs into endothelial cells. Differentiated cells expressed endothelial-specific markers such as KDR, FLT-1 and von Willebrand factor. The differentiated cells formed characteristic capillary-like structures. Using a chronic ischaemia model, Silva *et al.* administered MSCs intramyocardially into ischaemia-induced dogs [82]. After 60 days of MSC implantation, the dogs showed increased vascularity and reduced fibrosis.

Endothelial nitric oxide synthase (eNOS) is an attractive target for cardiovascular gene therapy. To determine the feasibility of Ad vector-mediated eNOS gene transfer into *ex vivo* expanded MSCs, Deng *et al.* [83] isolated rat MSCs and transduced them with an Ad5 vector encoding eNOS. The presence of eNOS protein in transduced rat MSCs was confirmed by immunohistochemical and western blot analyses. Intracavernosal injection of transduced rat MSCs increased the expression of eNOS in the corpus cavernosum. This shows that recombinant Ad vectors can be used to engineer *ex vivo* expanded MSCs and that high-level eNOS transgene expression can be achieved, again indicating the clinical potential of MSCs for the treatment of cardiovascular diseases.

4.3.4 Gene-modified mesenchymal stem cells to treat musculoskeletal disease —Viral vectors encoding BMPs 2 and 4 have been a recent research focus for the treatment of a variety of musculoskeletal defects. Lou *et al.* have documented MSC progenitor cell proliferation and differentiation *in vitro* and bone formation *in vivo* following transduction of such cells with an Ad vector encoding BMP2 [84]. In a study reported by Olmsted-Davis *et al.* [31], chimeric Ad vectors that contained an adenovirus Type 35 fibre (AdF35) encoding human BMP2 were used to transduce human MSCs. Such cells were then tested in an *in vivo* heterotopic bone formation assay. Mineralised bone was radiologically identified in muscle tissue implanted with Ad5F35-transduced human MSCs encoding BMP2, but not with control cells. In a related study, Gugala *et al.* compared the abilities of various human cell types with inherently dissimilar osteogenic potentials, including MSCs, to induce heterotopic bone formation following *ex vivo* transduction with two distinct Ad vectors encoding BMP2 [85]. Using NOD/SCID mice, transduced cells were injected intramuscularly following *ex vivo* Ad vector transduction. The nature and extent of heterotopic bone formation were analysed radiographically and histologically. At 14 days postinjection, abundant, highly mineralised bone was formed in mice injected with Ad5F35-BMP2-transduced cells. Substantially reduced bone formation was detected in mice injected with cells transduced with Ad5-BMP2. In all cell types studied, Ad5F35-BMP2 was more efficient than Ad5-BMP2 at providing adequate levels of BMP2 for efficient osteoinduction. In a comparative analysis involving Ad/RGD vectors and Ad vectors containing an unmodified fibre knob, MSCs were transduced using similar multiplicities of infection [32]. Rat MSCs transduced with Ad/RGD vectors encoding BMP2 produced higher amounts of BMP2 than cells infected with control Ad vectors encoding BMP2, and also differentiated towards the osteogenic lineage more efficiently *in vitro* than control cells. Furthermore, following *ex vivo* gene transduction, the potential for ectopic bone formation by the transduced MSCs *in vivo* was assessed. Ad/RGD-transduced MSCs exhibited greatly enhanced new bone formation compared to a control vector. These data suggest that Ad/RGD vectors provide powerful gene therapy tools for bone regeneration and other tissue engineering.

Blum *et al.* [86] evaluated the *ex vivo* genetic modification of rat MSCs using Ad, retroviral and cationic lipid vectors encoding human BMP2. *In vitro*, only MSCs modified with the Ad vector produced detectable BMP2 levels and demonstrated a statistically significant increase in endogenous alkaline phosphatase activity indicative of osteogeneic differentiation. The ability of genetically modified MSCs seeded on a titanium mesh scaffold to facilitate bone formation *in vivo* was also tested. In an orthotopic critical-size defect created in the rat cranium, bone formation was observed in all conditions with MSCs modified by the Ad vector, demonstrating a small but statistically significant increase in bone formation relative to MSCs transduced with control vectors. Implants in an ectopic location demonstrated minimal bone formation relative to the orthotopic location, with MSCs modified with cationic lipid-based vectors forming less bone than MSCs modified with retroviral or Ad vectors. This study was the first to compare three different gene delivery systems for the genetic modification of cells to produce osteoinductive factors for the purpose to enhance bone regeneration. In a study reported by Chang *et al.*, the clinical relevance of tissue engineering by integrating gene therapy

and polymer science to bone regeneration was examined [87]. Bilateral maxillary defects in miniature swine were bridged with a bioresorbable internal splint. Modified cells were prepared using Ad-BMP2-mediated gene transfer to expanded MSCs 7 days before implantation. BMP2-expressing cells displayed white solid bone formation after 3 months. These results show that *ex vivo* transduction of human MSCs using BMP2-encoding Ad vectors enhances autologous bone formation in the repair of maxillary defects.

Ex vivo strategies involving MSCs transduced with retroviral vectors encoding BMP4 were also reported. Gysin *et al.* [54] developed an efficient MoMLV-based retroviral system expressing the human BMP4 transgene. The bone formation potential of transduced cells expressing BMP4 was tested by embedding transduced stromal cells in a gelatin matrix that was then placed in a critical size defect in calvariae of syngenic rats. The defect area was completely filled with new bone in experimental rats after 4 weeks, whereas limited bone formation occurred in controls that included untransduced MSCs. More recently, Zhang *et al.* investigated the feasibility of increasing endosteal bone formation in mice by *ex vivo* gene therapy with MSCs transduced with a MoMLV-based retroviral vector expressing human BMP4. Transduced cells expressing BMP4 were injected into the femoral BM cavity and effects on bone were evaluated [88]. Direct intramedullary injection was successful and 2% of injected cells were present on average in the injected femur marrow cavity 24 h after injection.

In an exciting recent report, MSCs infected with AAV vectors encoding a dominant-negative collagen Type I protein have been used successfully to repair bones derived from individuals with the brittle bone disorder, osteogenesis imperfecta [89].

In an effort to develop *ex vivo* gene therapy for osteoporosis, Kumar *et al.* determined the efficiency of transduction of murine MSCs by recombinant AAV2 vectors carrying reporter genes or BMP2-encoding transgenes and determined their osteogenic potential in an immunocompetent mouse model for *ex vivo* osteoporosis gene therapy [34]. The data obtained highlight the potential usefulness of AAV-based vectors for *ex vivo* gene therapy of osteoporosis.

4.3.5 Gene-modified mesenchymal stem cells to impact tumour growth—Cancer gene therapy is the most promising and clinically most active field in gene therapy. Although previous experimental and clinical trials have brought forward some exciting results, the clinical benefits in general have been limited. As safety is a prerequisite to vector dissemination, tumour-specific targeting becomes crucial. Efficient vector dissemination in tumour masses and specific targeting of tumour cells are crucial for improving tumour-specific effects [90]. MSCs have been exploited to deliver genes encoding biological agents that impact tumour growth. Interferon (IFN)-β inhibits malignant cell growth *in vitro*. However, the therapeutic utility of IFN-β *in vivo* is limited by its excessive toxicity when administered systemically at high doses. Work reported by Studeny *et al.* [91] has shown that such toxicity effects can be reduced by delivering MSCs expressing IFN-β to tumours. Human MSCs were transduced with an Ad vector encoding human IFN-β. A SCID mouse xenograft model was used to examine the effects of injected MSC-IFN-β cells and of human recombinant IFN-β on the growth of MDA-MB-231 breast carcinoma cells and of A375SM melanoma cells *in vivo* and on survival. Co-culture of MSC-IFN-β cells with A375SM cells or MDA-MB-231 cells inhibited tumour cell growth as compared with growth of the tumour cells cultured without MSCs. Intravenous injection of MSC-IFN-β cells into mice with established MDA-MB-231 or A375SM pulmonary metastases led to incorporation of MSCs in the tumour architecture and, compared with untreated control mice, to prolonged survival. By contrast, intravenous injection of recombinant IFN-β did not prolong survival in the same models. Injected MSC-IFN-β cells suppressed the growth of pulmonary metastases, presumably through the local

production of IFN-β in the tumour microenvironment. Thus, MSCs appear to provide effective platforms for the targeted delivery of therapeutic proteins to cancer sites.

Stagg *et al.* [92] investigated whether IL-2 gene-modified MSCs can be used to mount a more effective immune response against the poorly immunogenic B16 melanoma cells. IL-2-producing MSCs mixed with B16 cells significantly delayed tumour growth in an IL-2 dose-dependent manner. Furthermore, matrix-embedded IL-2-producing MSCs injected in the vicinity of pre-established B16 tumours led to absence of tumour growth in 90% of treated mice. In a related study, Nakamura *et al.* [93] used gene-modified MSCs to inhibit malignant brain neoplasms. Primary MSCs isolated from Fischer 344 rats exerted inhibitory effects on the proliferation of 9L glioma cell *in vitro*. It was also found that MSCs inoculated into the contralateral hemisphere migrated towards 9L glioma cells through the corpus callosum. Intratumoural injection of MSCs caused significant inhibition of 9L tumour growth and increased the survival of 9L glioma-bearing rats. Gene modification of MSCs by infection with an Ad vector encoding human IL-2 augmented the antitumour effect and further prolonged the survival of tumour-bearing rats. Thus, gene therapy employing MSCs as a targeting vehicle may provide a new therapeutic approach for refractory gliomas.

MPCs have also been shown to foster expression of suicide genes and to support replication of oncolytic Ad vectors as potential anticancer agents. Pereboeva *et al.* evaluated the potential utility of such strategies with the intent to use them in a cancer therapy context [94]. By employing Ad/RGD vectors, MPC transduction resulted in efficient genetic loading of MPCs with reporter and anticancer genes. MPCs expressing thymidine kinase were able to exert a bystander killing effect on the human ovarian carcinoma cell line SKOV3ip1 *in vitro* following gancyclovir treatment. In addition, MPCs were able to support Ad replication, and thus can be used as cell vectors to deliver oncolytic viruses.

The role of multi-drug resistance (MDR) remains a major problem in the treatment of cancer with chemotherapeutic drugs. It is anticipated that gene therapy approaches to decrease the expression of such efflux transporters in tumour cells may increase the therapeutic efficacy of these drugs. In a recent study in mice, oral administration of a DNA vaccine encoding MDR-1 and carried by attenuated *Salmonella typhimurium* strains to secondary lymphoid organs, followed by the introduction of MDR-1-expressing colon or lung carcinoma cells, revealed a significant increase in lifespan of experimental animals [95]. The use of genetically modified MSCs expressing MDR-1 in conjunction with tumour-specific antigens may aid in mounting an enhanced antitumour effect.

5. Genetic manipulation of mesenchymal stem cells to promote their proliferation or differentiation

Unlike ESCs, adult MSCs, which lack telomerase activity [96], show defined *ex vivo* proliferation capability, reaching senescence and losing their multilineage differentiation potential after 34 - 50 population doublings in culture. Thus, it is crucial to have strategies available to prolong the replicative capacity of MSCs without impairing their multi-potentiality. Several studies have shown that forced ectopic expression of human telomerase reverse transcriptase (hTERT) in MSCs can dramatically extend their lifespan to > 260 population doublings, while maintaining their osteogenic, chondrogenic, adipogenic, neurogenic and stromal differentiation potential [97-99]. Serakinci *et al.* [100] investigated the neoplastic behaviour of such cells. A hTERT-transduced cell line, hMSC-TERT20, after 256 doublings showed loss of contact inhibition, anchorage independence and tumour formation in 10 of 10 mice. A related cell line, hMSC-TERT4, on the other hand showed loss of contact inhibition after 95 doublings, but did not exhibit anchorage independence and did not form tumours in mice. Both lines had a normal karyotype, but showed deletion of the Ink4a/ARF

locus. At later passage, hMSC-TERT4 cells also acquired an activating mutation in KRAS. In hMSC-TERT20 cells, expression of the cell cycle-associated gene DBCCR1 was lost due to promoter hypermethylation. This epigenetic event correlated with acquisition of tumourigenicity. These data suggest that the adult hMSCs can be targets for neoplastic transformation and have implications for the development of novel anticancer therapeutics and for the use of hTERT-immortalised MSCs in tissue engineering and transplantation protocols. In additions, caution must be exercised in using these immortalised MSCs, as they express higher levels of osteogenic lineage-specific genes, such as Cbfa1/Runx2, osterix and osteocalcin, compared with non-transduced MSCs, which could potentially compromise their ability to commit to other cell lineages [101].

As outlined above, transplantation of MSCs has been proposed as a strategy for cardiac repair following myocardial damage. However, poor cell viability associated with transplantation has limited the reparative capacity of these cells *in vivo*. Mangi *et al.* genetically engineered rat MSCs using *ex vivo* retroviral transduction to overexpress the prosurvival gene Akt1 (encoding the Akt protein) [102]. Transplantation of MSCs overexpressing Akt into the ischaemic rat myocardium inhibited the process of cardiac remodelling by reducing intramyocardial inflammation, collagen deposition and cardiac myocyte hypertrophy, regenerated 80 - 90% of lost myocardial volume, and completely normalised systolic and diastolic cardiac function. These observed effects were dose (cell number)-dependent. MSCs transduced with Akt1 restored fourfold greater myocardial volume than equal numbers of cells transduced with a control gene. Thus, MSCs genetically enhanced with Akt1 can repair infarcted myocardium.

Tsuchiya *et al.* [103] investigated chondrogenesis of cell-mediated therapy involving sox9 gene delivery as a new treatment regimen for cartilage regeneration. Sox9 is a member of the family of Sox (Sry-type HMG box) genes and plays a key role in chondrogenesis and skeletogenesis. A mouse sox9 cDNA was transfected into MSCs by lipofection and chondrogenic differentiation of these cells was evaluated. Transfected MSCs expressing sox9 were loaded into a diffusion chamber and transplanted into athymic mice to analyse *in vivo* chondrogenesis. Massive tissue formation of ~ 2 mm in diameter was visible in the chamber 4 weeks after transplantation. Histological examinations demonstrated that Type II collagen was present in the extracellular matrix of the mass, whereas type X collagen was not present. These results indicate that cell-mediated sox9 gene delivery could be a novel treatment strategy for cartilage damage.

Using a transgene delivery approach involving sequences corresponding to the Notch intracellular domain (NICD) and subsequent treatment with basic fibroblast growth factor, forskolin and ciliary neurotrophic factor, Dezawa *et al.* [104] demonstrated highly efficient and specific induction of cells with neuronal characteristics, without glial differentiation, from both rat and human MSCs. MSCs expressed markers related to neural stem cells after transfection with NICD-encoding sequences, and subsequent tropic factor administration induced neuronal cells. Further treatment of the induced neuronal cells with glial cell line-derived neurotrophic factor (GDNF) increased the proportion of tyrosine hydroxylase-positive and dopamine-producing cells. Transplantation of these GDNF-treated cells showed improvement in apomorphine-induced rotational behaviour and adjusting step and paw-reaching tests following intrastriatal implantation in a 6-hydroxydopamine rat model of Parkinson's disease. These results show that functional neuronal cells can be specifically generated from MSCs.

6. Expert opinion

MSCs are one of the most promising stem cells as a potential target for the clinical use of genetically engineered stem cells. One of the difficulties lies in the elusive nature of this unique

stem cell family that makes it difficult to confirm their stem cell properties and as the correlate to the HSCs in adult tissue. Another is the need to uniformly redefine the existing stem cell concept with the non-manipulated BM MSCs instead of the expanded mesenchymal cells that we are now also calling MSCs. Clearly the biology of the expanded MSCs does not provide the expected durable engraftment that stem cells are capable of providing. Rather, the *in vivo* biology of expanded cells suggests that they are more like a heterogeneous population of MSC-derived progeny produced after several generations *in vitro*. Furthermore, the expanded MSCs *in vivo* appear to be limited with both proliferation and differentiation potentials, and appear not to represent the original non-manipulated self-renewing multipotent or MAPC population from BM. The important features currently lacking in the MSC system are their identification, biology and functional distinction between their progenitor cell lineages and their terminally committed progeny. All are needed in order to understand the hierarchal origin of the adult MSC system so that progenitor cells are named in a uniform manner according to their function (i.e., types of colonies they can generate).

The relatively low frequency and functional heterogeneity of MSC-derived progenitor cells from BM, historically and collectively represented as colony-forming units fibroblast (CFU-F), support the basic concepts that structure the hierarchal model of the HSC system. Providing MSC and progenitor cell populations capable of durable engraftment and as potential targeted vehicles for gene therapy hinges in defining the hierarchal position and biology of the expanded MSCs. The 'Achilles' heel' of the current concept of MSCs may lie in the extensive proliferation capacity that we may be naively taking advantage of, where, in fact, extensive passages may be producing great waves of cells terminally ready to differentiate, but can not grow anymore or anywhere [105]. Thus, the lack of telomerase activity in MSCs, for example, may not be a paradox after all. The stem cell nature of the original MSC and progenitor cell population may be lost with current expansion systems that appear to give great promise *in vitro*, but without *in vivo* efficacy. Therefore, it should not be too surprising that their *in vivo* engraftment potential may no longer exist.

Although there has been extensive characterisation of differentiation potential of MSCs *in vitro*, there is little understanding on the possible different mechanisms of the interactions of fresh or non-manipulated versus expanded MSCs with tissue microenvironments *in vivo*. There appears to be at least two mechanisms involved. True engraftment processes involve homing, clonal expansion or nesting, followed by terminal differentiation. The other mechanism involves genetic integration events caused by cell to cell fusion between donor and recipient cells, which incidentally may account for the low frequency of expanded donor mesenchymal cells in various tissues *in vivo*. Thus, durable engraftment mechanisms expected from fresh or non-manipulated MSCs are predicted to assure sustained and directed gene expression to a specific tissue. In contrast, fusion may be the primary mechanism in expanded MSCs, and may only involve nonspecific entrapment or scattered lodgement, particularly in organs of the reticulo-endothelial system, as seen in lungs, liver, spleen, gut and BM. At present, expanded MSCs appear to result in only a transient production of the gene product with a low frequency of undirected donor cells that are scattered nonspecifically in tissues. Studies are needed to understand the different biology of fresh or non-manipulated versus expanded MSCs and their interaction with tissue microenvironments. The stem cell for the haematopoietic and mesenchymal systems remains elusive, as there is no known marker that identifies exclusively either the HSC, haematopoietic progenitor cells, or the MSC; yet the biology of the HSC has served to identify its presence among a heterogeneous population of $CD34^+$ progenitor cells and other cells that also express CD34, such as endothelial cells and mast cells. Similarly, studies utilising antibodies to known MSC-associated markers, such as nerve growth factor receptor, CD49a or STRO-1 [106-108], have shown that progenitors, CFU-F, to MSCs can be enriched. Further discovery of novel surrogate markers, may serve in a similar fashion as the CD34 antigen has as the surrogate marker for the haematopoietic stem cell. Fresh MSC isolates

from BM, for example, may serve as a model population of unmanipulated MSCs, whose *in vivo* and *in vitro* biological activity may be compared to that of serially passaged MSCs. These studies may help define expansion conditions that maintain a yet earlier population of MSCs that can self-renew, that is, MAPCs, while producing a heterogeneous population of relatively more mature progenitors, that is, CFU-F. When MAPCs are transplanted as single cells into blastocysts, their progeny can repopulate all tissues and organs including all lineages in the CNS, and similarly differentiate into mesenchymal, epithelial, endothelial and neuronal lineages *in vitro* [109]. This early cell is relatively well-characterised and can be distinguished functionally from MSCs and CFU-F. Further studies are needed to better understand the hierarchical relationship between MAPCs, CFU-F and MSCs. More importantly, identification of the *in vivo* correlates may provide a better understanding of outcomes in clinical studies.

In conclusion, novel strategies that allow the isolation of M-MSCs and their genetic manipulation without interfering with self-renewal and differentiation processes are critical to assure both durable engraftment and long-term therapeutic effects of genetically engineered MSCs.

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