Advanced Drug Delivery Reviews xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Mesenchymal stem cells for the sustained *in vivo* delivery of bioactive factors ☆

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ARTICLE INFO

Article history:
Received 7 September 2010
Accepted 24 September 2010
Available online xxxx

Keywords:
Human mesenchymal stem cells
Gene delivery
Cytokines
Enzyme replacement
Hypoxia
Tissue repair
Xenotransplantation
Translational studies
Clinical trials

ABSTRACT

Mesenchymal stem cells (MSC) are a promising tool for cell therapy, either through direct contribution to the repair of bone, tendon and cartilage or as an adjunct therapy through protein production and immune mediation. They are an attractive vehicle for cellular therapies due to a variety of cell intrinsic and environmentally responsive properties. Following transplantation, MSC are capable of systemic migration, are not prone to tumor formation, and appear to tolerize the immune response across donor mismatch. These attributes combine to allow MSC to reside in many different tissue types without disrupting the local microenvironment and, in some cases, responding to the local environment with appropriate protein secretion. We describe work done by our group and others in using human MSC for the sustained *in vivo* production of supraphysiological levels of cytokines for the support of cotransplanted hematopoietic stem cells and enzymes that are deficient in animal models of lysosomal storage disorders such as MPSVII. In addition, the use of MSC engineered to secrete protein products has been reviewed in several fields of tissue injury repair, including but not limited to revascularization after myocardial infarction, regeneration of intervertebral disc defects and spine therapy, repair of stroke, therapy for epilepsy, skeletal tissue repair, chondrogenesis/knee and joint repair, and neurodegenerative diseases. Genetically engineered MSC have thus proven safe and efficacious in numerous animal models of disease modification and tissue repair and are poised to be tested in human clinical trials. The potential for these interesting cells to secrete endogenous or transgene products in a sustained and long-term manner is highly promising and is discussed in the current review.

Contents

1. Overview

Mesenchymal stem cells (MSC) are a promising tool for cell therapy, either through direct contribution to the repair of bone, tendon and cartilage or as an adjunct therapy through protein production and immune mediation. They are currently being tested in clinical trials for such diverse applications as myocardial infarction, stroke, meniscus injury, limb ischemia, graft-versus-host disease and autoimmune disorders. To date, MSC have been extensively tested and proven effective in pre-clinical studies for these and many other disorders, including neurodegenerative diseases [1–5].

MSC are an attractive vehicle for cellular therapies due to a variety of cell intrinsic and environmentally responsive properties. Molecularly equivalent MSC have been successfully harvested from several locations, including the marrow space and various fat pads, with minimal patient discomfort. Once acquired, MSC are easily isolated

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Stem Cell Gene Manipulation and Delivery as Systemic Therapeutics".

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0169-409X/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.addr.2010.09.013

and characterized *in vitro* and exhibit rapid proliferation with minimal senescence through multiple passages [6,7]. In contrast to hematopoietic progenitors, this rapid expansion does not dilute the capacity for self-renewal and provides the unique opportunity to easily introduce a target gene of interest through a variety of methods [8]. Also, in contrast to many other potent progenitor populations, MSC require minimal culture conditions and media, as they produce many of their own essential growth factors to self-sustain through autocrine and paracrine mechanisms. Following transplantation, MSC are capable of systemic migration [8–13], are not prone to tumor formation [14], and, in fact, appear to tolerize the immune response across donor mismatch [5,15]. These attributes combine to allow MSC to reside in many different tissue types without disrupting the local microenvironment and, in some cases, responding to the local environment with appropriate protein secretion [16–18].

Due to their ability to reside within many tissue environments, MSC have been pursued as a therapeutic intervention in several models of chronic and acute injury. The factors governing the duration of their residence in the damaged tissue are not yet fully understood and seem to differ between injury types. In cases of acute injury or inflammation, MSC respond to the injury robustly but only transiently and do not become a stable part of the repaired tissue or vasculature to a significant degree. The same data has been obtained in large animal models and appears to be independent of immune rejection. In the transient engraftment/acute injury models, data suggest that their efficacy relies on paracrine actions rather than differentiation and direct contribution to the damaged tissue. MSC are home to the injured area, in particular to hypoxic, apoptotic, or inflamed areas, and release trophic factors that hasten endogenous repair. These secreted bioactive products can suppress the local immune system, enhance angiogenesis, inhibit fibrosis and apoptosis, and stimulate recruitment, retention, proliferation and differentiation of tissue-residing stem cells. Paracrine effects exerted by MSC are distinct from the classical model of direct differentiation of stem cells into the tissue to be regenerated. Some current studies aim to enhance these paracrine effects through forced over-production of various paracrine elements to further hasten the endogenous repair processes [5,19–22].

In contrast to the short-term survival patterns in the acute injury setting, when MSC are infused intravenously into immune-deficient mice that have low level systemic damage from irradiation, or a chronic disease, the cells migrate through all tissues in a relatively evenly dispersed and long-lasting manner [8,12,13,23]. In our laboratory, we have recovered genetically engineered human MSC from numerous organs of mice at timepoints from 6 to 18 months post-transplantation, with continued expression of transgene products for the duration of the experiments [8,12,13,23]. *In situ* examination revealed a lack of scarring or inflammation around these cells, and explant cultures have demonstrated that these MSC retained the ability to proliferate once released from the extracellular matrix constraints of their resident tissues [12,23]. The current work in our laboratory is focused on determining the molecular adaptations that occur in these tissue-resident MSC and how this microenvironmental responsiveness can be efficiently exploited in chronic injury therapeutics.

These specialized characteristics of MSC have made them a promising vehicle for cell-based therapeutic intervention of acute and chronic injury, through direct integration into damaged tissue, as well as trophic support and immune mediation during endogenous regeneration and repair mechanisms. They are easily harvested and expanded, are amenable to genetic manipulations, and have a decade-long record of biosafety data *in vivo* [14,21]. Therefore, while the factors that govern the duration of residence of MSC in different tissues during acute versus chronic disorders must be further delineated, the potential for these cells to secrete endogenous or transgene products in a sustained and long-term manner is highly promising and is discussed in the current review.

2. Characterization and utility of mesenchymal stem cells

Mesenchymal stem cells (MSC) were originally identified by Friedenstein and his colleagues as the primary transplantable component of the bone marrow microenvironment necessary for the maintenance of definitive hematopoiesis [24]. This original description established several characteristics of this cell type, which are still the primary hallmarks of MSC today, in defiance of 3 decades of technological advances in scientific research and an expanding knowledge base on the nature of stromal cell influence in the development of blood systems. Specifically, Friedenstein defined mesenchymal stem cells as fibroblastic mesodermally derived cells that are adherent and clonogenic [24–26]. This clonogenic expansion was measured in an assay termed the CFU-F, or colony forming unit-fibroblast, and presumed to be the mechanism through which the marrow microenvironment could survive through myeloablative conditioning and facilitate hematopoietic reconstitution. Subsequent experiments by Friedenstein and his collaborators established *in vivo* evidence using a rat transplant model to demonstrate the importance of the stromal component in hematopoiesis [24,27]. These early findings led to the increased examination of the marrow microenvironment, its role in the propagation of hematopoiesis, and the mechanisms by which these cells were able to elude cell death throughout regimens of myeloablation.

The rapidly dividing adherent myofibroblastic cells from the human bone marrow microenvironment were previously referred to as "stroma," but the better term "mesenchymal stem cells" reflects their capacity to differentiate into multiple tissues, bone, cartilage, tendon, fibroblast, fat, and muscle [28–30]. The term MSC can also be used to denote marrow stromal cells and the terms are often used interchangeably, although true mesenchymal stem cells, the most primitive subset, are likely rare in the marrow stromal cell myofibroblastic layer. Unfortunately, the phenotype of the most primitive MSC compartment has not yet been clearly defined, and the phenotype may vary with culture or expansion conditions, as can be seen with hematopoietic stem cells, making it difficult to standardize. We and other researchers have grown the cells out of bone marrow spicules [8] and marrow samples based on their ability to adhere to plastic and to rapidly expand in minimal medium. Description of some of the markers that are found on MSC has been done. However, these markers may or may not be on overlapping subsets, and there has been no systematic analysis of differentially sorted populations, as has been done with human hematopoietic stem cells. Therefore, a major goal in the field is to characterize the most primitive subsets of human MSC and to define their functions and differentiative capacity *in vitro* and *in vivo*.

Phenotypic characterization of the most primitive subset of MSC is therefore still elusive, and prospective identification of a homogenous, self-renewing and pluripotent population has been hampered by the diversity of cell populations referred to in the literature as MSC. Conventional assays such as the CFU-F do not sufficiently define the cell type responsible for observed multi-lineage differentiation and, in fact, cannot adequately rule out the possibility that multiple cell types within the marrow space are contributing. To address this inconsistency, the International Society for Cellular Therapy has recently established minimal criteria for defining multipotent mesenchymal stromal cells [31]. These basal attributes include the ability to adhere to plastic under normal cell culture conditions, to express a distinct set of 3 cell surface antigens (CD105, CD73, and CD90) while not expressing antigens indicative of other cell lineages, and to differentiate into adipocytes, osteoblasts, and chondroblasts under specific conditions. This set of minimal guidelines has served to allow a basis of comparison between the results of different investigators and has allowed a more focused investigation into the clinical utility of stromal stem cells.

Investigation into the physiological relevance of mesenchymal stem cells has revealed several characteristics that make them an appealing target for use in cell- or gene-therapy-based interventions. Kuznetsov

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and colleagues described several morphologically distinct cell populations within their isolations, which were found to have varying clonogenic potential when transplanted into animal recipients [32]. These differences have since been attributed to a surprising amount of lineage differentiation diversity from a cell population resident in the marrow space. When transplanted under the kidney capsule of rats, for example, individual MSC clones were found capable of forming a complete marrow ossicle [32,33]. These *de novo* ossicles contained osteogenic cells interfacing with traditional marrow sinusoidal cells and adipocytes and were capable of supporting hematopoiesis. Upon closer examination, they discovered that these functional marrow sinusoids were not generating their own hematopoietic progenitors but rather had recruited host origin HSC to the transplant site. At once, these experiments implied that marrow stromal stem cells not only were able to completely recapitulate an environment for the development of hematopoiesis but also were immune-privileged and compatible to interact with many diverse cell types without rejection. Furthermore, these experiments imply a mechanism for the active recruitment of hematopoietic progenitors to areas hospitable for expansion, a mechanism indispensable to post-myeloablative transplant repopulation and hematopoietic reconstitution.

In the marrow cavity, it seems unlikely that all MSC lining the marrow sinusoidal space are involved equally in the processes of self-renewal, hematopoietic support, and secretion of autocrine and paracrine factors [34] but rather that distinct subsets of MSC function in tandem to maintain the microenvironmental niche. The generation of the type of diverse microenvironment as seen by Kuznetsov et al. from a single MSC clone [32,33] requires massive cell proliferation, and upon examination *in vitro*, mesenchymal stem cells have been shown to undergo as many as 25 self-renewing replications without detectable morphological change or loss of lineage potentiality. This potential for self-renewing proliferation and generation of multi-lineage progeny, coupled with the immunological tolerance initially suggested by the kidney capsule transplant model and later confirmed by many other studies [15], has presented MSC as a highly attractive target for clinical therapeutic intervention.

3. Systemic cytokine production from genetically engineered human MSC

The first use of MSC in the gene therapy field was as a supportive monolayer for hematopoietic stem and progenitor cells [35]. During the gene transduction of hematopoietic stem cells, the stromal layer was found to improve survival through a “coculture” method [36]. As this practice was improved, it was discovered that in addition to providing *ex vivo* support during the gene transfer or expansion phase of HSC-directed therapy, the MSC themselves could be engineered to provide increased human cytokine support when cotransplanted with the HSC [23,37]. As more becomes known about the biological characterization of bone-marrow-derived MSC, more interest is generated in using these cells for cell- or gene-directed therapy. Special interest, in particular, has evolved for using MSC as a protein delivery vehicle following gene therapy modification. From a very small marrow aspirate, human mesenchymal stem cells are easily isolated and will rapidly begin clonogenic expansion *in vitro*. When compared to hematopoietic stem cells, which can be harvested in a similar fashion, MSC cultures do not have the expensive cytokine and growth factor requirements since they produce many autocrine factors on their own [38]. Also, unlike traditionally used HSC, there is no defined hierarchy of MSC development, either *in vitro* or *in vivo*, which describes specific conditions for the maintenance of the most potent cell type. Following the transduction of MSC monolayers, relatively equivalent expression of the transgene product can be achieved throughout the culture.

In contrast to HSC, retroviral engineering did not seem to impair the ability of MSC to proliferate, self-renew, migrate post-transplantation, or differentiate appropriately [39–41]. Furthermore, these studies suggested that MSC have fewer complications regarding the insertion of virally delivered transgenes. Whereas HSC seem to maintain a highly quiescent pool of true stem cells [42–47] that are resistant to retroviral transduction, MSC seem to have no comparable metabolic barrier. Several studies have verified this finding, demonstrating that MSC can be efficiently and durably transduced without intensive labor and that this transgene expression is maintained throughout lineage differentiation and without compromising the proliferation rate or quality of progeny [48–51]. The clonal analysis of the resultant cell populations showed wide variation; however, some clones contained several thousand copies of transgene RNA per cell and were able to maintain this expression for up to 6 months post-transduction [50]. The examination of the starting cell population further showed that nearly 90% of all cells capable of producing colony-forming unit-fibroblast (CFU-F) colonies were transduced using standard transduction procedures [50]. In comparison to HSC transduction, this approach represented an astounding and intriguing finding for cellular therapy and genetic engineering.

Interestingly, after transplantation, the human MSC do not migrate effectively to the bone marrow stromal compartment. However, Bubnic et al. demonstrated that W/Wv mouse-derived marrow stromal cells engrafted into the marrow compartment and enhanced early erythropoiesis in unconditioned Sl/Sld murine recipients. The engraftment of donor stromal cells reached levels of up to 1.0% of total marrow cells 4 months post-transplantation [52]. This report was interesting because transplanted MSC do not usually replace the endogenous microenvironment, even after conditioning [53,54]. This report therefore suggests that the defect in the stromal compartment of the Sl/Sld mice played a role in permitting MSC engraftment in the marrow compartment. Almeida-Porada et al. demonstrated that the cotransplantation of both autologous and allogeneic human bone-marrow-derived HSC and MSC in a fetal sheep model resulted in higher levels of long-term engraftment of human hematopoietic cells in the bone marrow of the chimeric animals during gestation and after birth [55,56]. By using marked MSC, they also demonstrated that injected stromal cells engrafted and retained function within the sheep marrow. This study suggests that the fetal microenvironment could also be more permissive to allowing MSC engraftment. These reports could provide clues as to how to better replace at least a portion of the microenvironment for *in vivo* studies or clinical indications.

In the majority of the MSC transplantation studies reported to date, the transplanted cells lodge in multiple organs and continue to secrete their transgene products from those locations throughout numerous tissues [8–13]. Noort et al. identified a population of MSC derived from a human fetal lung, which promoted the engraftment of cotransplanted umbilical cord blood CD34(+) cells in bone marrow, spleen, and blood. Again, no MSC were found in the marrow compartment, suggesting that the mechanisms by which the engraftment was enhanced did not require homing of MSC to the bone marrow [57].

Angelopoulou et al. showed that the cotransplantation of human mesenchymal stem cells with HSC enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice [58]. The cotransplantation of genetically engineered human MSC solved a problem that had hampered the field's use of human-to-murine xenotransplantation prior to 1988: the lack of cytokines cross-reactive to human stem cells in the mice. In that year, Kamel-Reid et al. described the bnx/hu model, in which the immune-deficient mice were injected with human interleukin 3 (IL-3), and human progenitors could survive for several months, with a primarily myeloid differentiative capacity [59]. To avoid expensive and time-consuming injections of cytokines every 48 hours, as Kamel-Reid et al. had described [59], our group cotransplanted human marrow stromal cells (MSC) engineered to secrete human IL-3, in addition to their endogenous human growth

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factors [49]. The species specificity of IL-3 allowed the support of human hematopoietic cells in the mice without perturbing murine hematopoiesis. Jiang et al. later confirmed that the cotransplantation of stromal cells engineered to secrete IL-3 with T cell depleted marrow grafts improved hematopoiesis following allogeneic transplantation in mice [60].

In our group's cotransplantation system, gene-transduced human CD34+ progenitor cells were transplanted into immunodeficient mice after *in vitro* binding to primary human bone marrow (BM) stromal cells engineered to produce human interleukin-3 (IL-3). The IL-3-secreting stroma produced sustained supraphysiological circulating levels of human IL-3 for at least 4 months in the mice. HuIL-3 levels were 209.4 ± 15.5 pg/ml at 1 month and 35.5 ± 5 at 6 months (N = 7; Fig. 1). Levels at six months remain higher than the normal human physiological levels of IL-3 (2 pg/ml) [61]. There were significant influences from the IL-3-expressing MSC on the development of cotransplanted human hematopoietic stem cells (HSC). HuIL-3 allowed huHSC survival in beige/nude/xid (bnx) mice, with little engraftment of hematopoietic cells observed in control animals.

In related studies, we coinjected human bone-marrow-derived hematopoietic CD34+ cells with human marrow stromal cells engineered to secrete human IL-2, IL-7, stem cell factor (SCF) or FLT3 ligand (FL), with and without IL-3. No single factor other than IL-3 supported sustained human hematopoiesis in the mice. The use of IL-2 was discontinued due to cross-reaction and adverse effects on the murine hematopoietic system, as well as vascular leaking. The coexpression of SCF or FL with IL-3 had no overt effect on human hematopoiesis. The production of both human IL-3 and IL-7 in the mice supported the extrathymic development of human T lymphocytes for 6–8 months [62], but no B cells, myeloid cells, or colony-forming progenitors were detected in those mice, whereas they developed in mice transplanted with IL-3 producing MSC alone. This result demonstrated the capacity of the engineered MSC to skew hematopoietic development from the hematopoietic stem and progenitor cells in the mice [49].

Li et al. later showed that cytokine-transduced murine bone marrow stromal cell lines promoted immunohematopoietic reconstitution in mice after cotransplantation with HSC. Murine MSC were transfected with the murine IL-3 and/ or IL-2 genes and then injected into lethally irradiated C57BL/6 mice with allogeneic T cell depleted bone marrow. The cytokine-transduced stromal cells significantly increased the numbers of hematopoietic progenitors and lymphocytes derived from the cotransplanted HSC [63].

A limitation to the study of human hematopoietic development in immune-deficient mouse models is the lack of maturation of human red blood cells, although BFU-E are maintained. Mature red cell development in the mice could provide a useful model to study gene therapy for globin disorders *in vivo*. To try to obtain maturation from the BFU-E stage, we cotransplanted human marrow stromal cells secreting human erythropoietin (Epo) and IL-3 into mice. An increase in hematocrit from 40%–45% to 80%–85% resulted, with the production of human and murine red blood cells. Unfortunately the production of Epo was too effective using this model, since all mice (N = 9) suffered strokes, displayed paralysis and died within three weeks [49]. We were able to refine this system by reducing the level of human Epo expression through the use of alternate MSC delivery methods, such as a biochamber or neo-organoid, which limited the systemic Epo levels by restricting the number of Epo-producing MSC [64] (Fig. 2). These studies are illustrative of the effects that can be achieved from the supraphysiological expression of cytokines from MSC, which can produce and secrete transgene products at relatively high levels.

MSC genetically engineered to secrete cytokines and other growth factors have been used recently in animal models of various tissue repair studies. Kucic et al. showed that Epo-producing MSC were useful in the long-term correction of renal failure-induced anemia [65,66]. They next demonstrated that the cotransplantation of insulin-like growth factor I (IGF-I)-overexpressing MSC improved the MSC-based gene therapy of anemia by providing paracrine support to Epo-secreting MSC [67]. Mice were rendered anemic by right kidney electrocoagulation and left nephrectomy. MSC engineered to express Epo were subsequently implanted subcutaneously in a bovine collagen matrix, in combination with MSC expressing IGF-1 or MSC alone in mice with renal failure. In mice that had received MSC–Epo coimplanted with MSC–IGF, the hematocrit elevation was enhanced compared with control mice, and the heart function was also improved. Their strategy of the coimplantation of MSC–IGF with MSC–Epo therefore represents a promising strategy for improving cell-based gene therapy of renal anemia [67].

Xu et al. examined the efficacy of murine-MSC-based angiopoietin-1 gene delivery for acute lung injury [68]. Angiopoietin-1 (Ang1) is a critical factor for endothelial survival and vascular stabilization. In their *in vivo* mouse model, LPS-induced lung injury was markedly alleviated in the group treated with MSC engineered to express Ang1 compared to groups treated with MSC or Ang1 alone. Their results indicated that

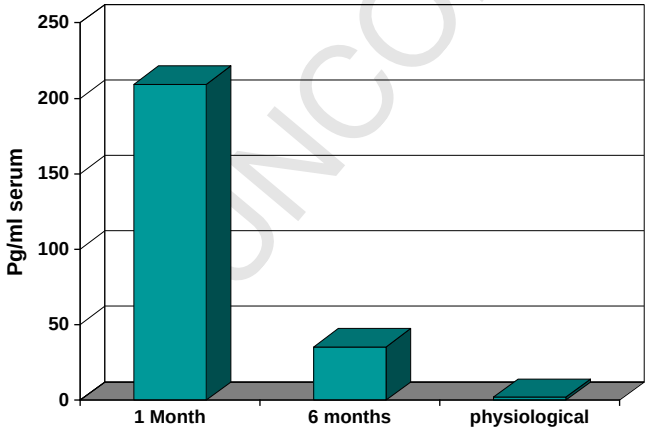

Fig. 1. IL-3 levels were assessed in the serum of immune-deficient mice one and six months after cotransplantation with engineered human mesenchymal stem cells. At one and six months post-transplantation, blood was collected from the tail vein of the mice and the level of HuIL-3 in each serum sample was assessed (N = 7). Serum levels were calculated by linear regression analysis in comparison to a standard curve generated from dilutions of recombinant HuIL-3. Levels at six months remained higher than the normal human physiological levels of IL-3 (2 pg/ml).

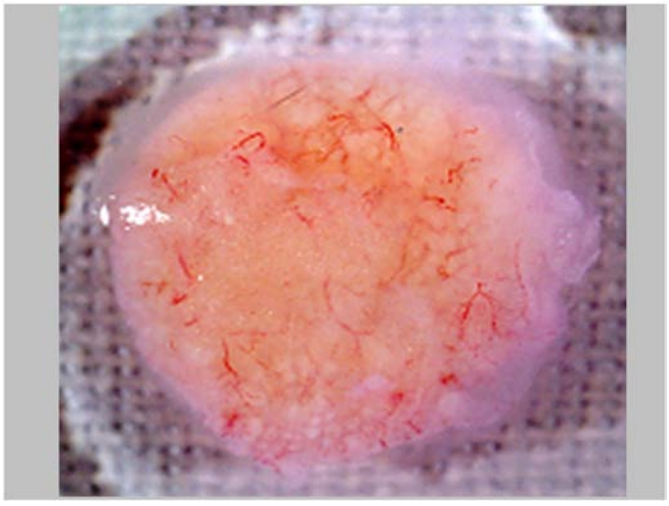

Fig. 2. Epo-expressing human BM–MSC organoid implanted into nude/NOD/SCID mice on a biodegradable matrix. The MSC-based tissue is highly vascularized at the time of harvest, one month after implantation.

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MSC-based Ang1 gene therapy could potentially be developed as a novel strategy for the treatment of acute lung injury [68].

In addition to these specific examples of growth-factor-expressing MSC in tissue repair, other fields of tissue injury repair have been explored, including but not limited to revascularization after myocardial infarction (reviewed in [69,70]), regeneration of intervertebral disc defects and spine therapy (reviewed in [71,72]), repair of stroke (reviewed in [73]), therapy for epilepsy (reviewed in [74]), skeletal tissue repair (reviewed in [19]), chondrogenesis/knee and joint repair (reviewed in [75]) and neurodegenerative diseases (reviewed in [1,76]). Genetically engineered MSC have thus proven safe and efficacious in numerous animal models of tissue repair and are poised to be tested in human clinical trials.

4. Systemic production of enzyme from human MSC

While there is now ample evidence for the utility of MSC as trophic support for other cell types, both *in vitro* and *in vivo*, a related area of research has emerged to examine the use of MSC as a stand-alone therapeutic *in vivo*. The ability of MSC to traffic systemically, coupled with their ease of transduction, presents a favorable platform for the delivery of a therapeutic protein in models of genetic insufficiency. Of particular interest is the clinical intervention of the so-called orphan diseases, which affect so few individuals nationwide that robust protein replacement regimens have not yet been developed through pharmaceutical pipelines. These treatments are further complicated by their rare incidence, as appropriate donor matches for bone marrow transplant may be familial carriers.

Lysosomal storage disorders (LSDs) present an appealing model for studying the efficacy of MSC in cell- and gene-therapy-based treatment. This group of disorders encompasses greater than 45 distinctly characterized diseases, most of which result from enzyme deficiencies in the cellular breakdown of waste products through lysosomes. Mucopolysaccharidosis type VII (MPSVII), in particular, results from the lack of beta-glucuronidase (GUSB) production caused by a variety of genetic mutations within chromosome 7. The loss of enzyme function in these disorders leads to the progressive accumulation of waste material within cells throughout the body, causing multi-system failure and profound developmental defects. Therapeutic intervention of MPSVII and other LSDs can be achieved by taking advantage of cross-correction through an intracellular transport system of these enzymes via mannose/mannose-6-phosphate receptors. In many cases, cross-correction can be achieved with the introduction of single-digit percentage-of-normal amounts of enzyme [77].

An alternative strategy to allogeneic bone marrow transplantation for lysososmal storage disorders is an autologous transplant using gene therapy to correct the deficiency in the patient's own cells and then to re-introduce them to the patient. This approach bypasses the need for a matched allogeneic donor, as the therapy uses the patient's own corrected cells. Previous work with the MPS diseases has reported good success with gene therapy approaches in a variety of models and transplant schematics. Initial investigations proved the efficacy of human GUSB in the correction of animal models [78,79], and researchers continued to examine alternative cellular targets for therapy, including hematopoietic progenitors [80,81] and skin fibroblasts [82,83]. Hematopoietic progenitors were primarily re-introduced via traditional BMT procedures, whereas therapies involving fibroblasts met with greater success as implantable neo-organs [82,83] within the peritoneal cavity. While both approaches achieved moderate success and global distribution of enzyme, effective correction was not seen in all organs, thus prompting inquiry into alternative methods to effectively target specific organ systems.

The translation of these preliminary data into clinically relevant information is a current area of focus in the field of gene therapy, in general, and is an active area of research for the MPSVII disease. The most promising translational data to date have come from the lentiviral engineering of human hematopoietic progenitors, followed by transplantation into an immune-deficient mouse model of MPSVII [84]. This study by Hofling et al. showed a systemically efficient cross-correction of the disease and was followed up by an elegant study utilizing the lentiviral gene-replacement therapy on HSC from a human MPSVII patient, followed by transplantation into the murine MPSVII model [85]. Similar to the prior study, the human donor cells were able to efficiently repopulate the murine hematopoietic system, showing the correction of disease with no adverse effects of lentiviral transduction [85]. In spite of these promising results, gene-replacement therapy in the hematopoietic system is not without drawbacks. The transduction efficiency of pseudotyped lentiviral vectors is not 100%, which, when coupled with the colony-forming efficiency of the best CD34+ progenitor population, leaves much room for improvement.

While this limited success through various methods was encouraging, each paradigm came with a unique set of limitations. Given the multi-system defects and the need for systemic delivery of a protein, MSC transplantation seemed a promising area of research. With regard to MSC engraftment within the model, while there are no assays complementary to the marrow replacement seen in an HSC transplant, transplanted MSC have been shown to disperse to a wide range of tissues following a variety of routes of administration [41]. Following this differential repopulation into multiple tissue beds, the transplanted MSC were found to persist and maintain viability at least 75 days post-transplantation. Explant cultures also demonstrated that the MSC retained a limited ability to proliferate again once the restrictions of extracellular matrix contact within the tissues were removed [41].

Our group further evaluated the potential for human adipose-derived mesenchymal stem cells to traffic into various tissue compartments in the NOD/SCID/MPSVII mice [12]. For up to 75 days post-transplantation, donor-derived cells were observed in multiple tissues, with similar levels across the various administration routes and independent of transduction parameters. Tissue localization studies showed that the primary MSC did not proliferate extensively at the sites of lodgment (Fig. 3) [12]. Human adipose MSC therefore represent a population of stem cells with a ubiquitous pattern of tissue distribution after administration, similar to the traditional bone marrow resident cells. However, a cellular therapy solution for MPSVII and other enzyme deficiency disorders will require not only widespread distribution of cells [86] but also sufficient enzyme production and secretion by the transplanted donor cells.

In order to evaluate the potential for MSC in treating these diseases, we performed extensive assays *in vitro* to quantify the GUSB enzyme production of MSC in comparison to other cell types, as well as the increased production from lentiviral transduction for constitutive expression [13]. A corollary goal of this study was to examine the amount of enzyme secreted from MSC to be available for cellular re-uptake and cross-correction. While previous reports had used a variety of cell types to deliver GUSB, direct measurements of these parameters had not been performed and compared between cell types. Our results demonstrated that MSC produce and secrete minimal amounts of GUSB natively, in a comparable range with other fibroblastic cell types. Conversely, following lentiviral transduction, primary human MSC produced nearly 100-fold more GUSB per cell. In comparison with primary human hematopoietic progenitors (total lineage depleted umbilical cord blood-derived HPC), this results represents a 200-fold greater production and approximately 158% more GUSB than parallel cohorts of normal human skin fibroblasts. Consequently, this increased total GUSB production results in a similarly increased amount of secreted enzyme [13]. The observed *in vitro* cross correction in those studies demonstrated that the engineered human MSC were producing and secreting

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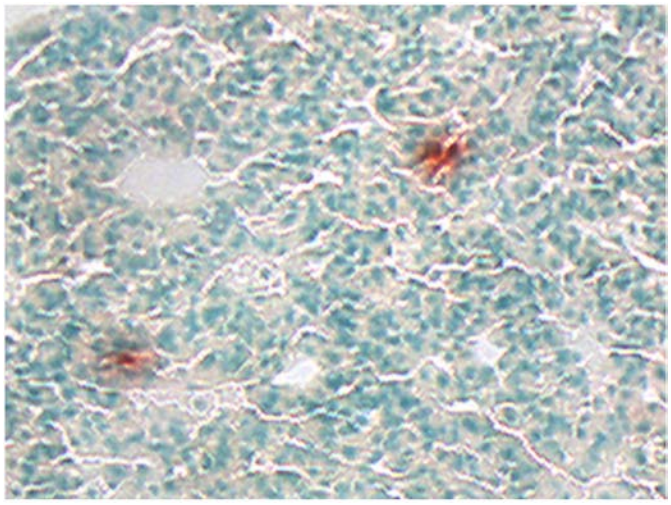

Fig. 3. Tissue section from nonobese diabetic/severe combined immunodeficient/ MPSVII immunodeficient mouse transplanted intravenously with human adipose-derived mesenchymal stem cells. This strain is null for the enzyme beta-glucuronidase (GUSB). Tissue sections were stained for enzyme activity using a substrate (red) to identify normal GUSB levels in transplanted human MSC and then were counterstained with methyl green. An example of MSC localization in lung is shown.

conformationally correct enzyme with proper phosphorylation. Therefore, we demonstrated that the constitutive overexpression of GUSB did not lead to faulty protein processing or excretion [13].

Having demonstrated the ability of MSC to both produce and secrete supraphysiologic levels of enzyme *in vitro*, the next step was to ensure the fidelity and maintenance of this process *in vivo*. The histologic examination of multiple murine tissues post-transplantation confirmed the presence of GUSB and successful cross-correction [13]. The histopathologic scoring of these tissues showed significant reduction in the amount of accumulated GAG from cellular cross-correction, with biochemical staining in many organs nearly as intense as enzymatically normal NOD/SCID animals [13]. This finding was confirmed through the direct measurement of enzyme activity using tissue homogenates and was further validated using measurements of secondary enzyme elevations [13]. Further validation for the efficacy of MSC to deliver enzymes in this model of MPS was provided from the pathological examination of tissues from mice that had received GUSB-expressing MSC. The tissues from those mice, in comparison to untreated controls, had a marked reduction in lysosomal storage in nearly every organ system evaluated, including previously refractory targets such as kidney and brain [13].

The quantitative PCR analysis of biochemically corrected organ lysates showed that the correction was not due to a significantly different repopulation by donor cells in the virally engineered cohorts and that the biochemical correction observed was due to the cross-correction of murine cells. Calculations to estimate the total number of human cells present at the 4-month timepoint indicated no significant difference in engraftment levels between engineered and unmanipulated cells. Further investigation revealed that the increased production of lentiviral-expressed GUSB had allowed serum levels in treated animals to approach nearly 40% that of wild type MPSVII mice. This level of circulating GUSB represents a significant improvement over the levels attained in any other cellular therapy to date and is the driving force behind the correction observed in these animals [13].

The results of these experiments indicate that MSC may have utility in therapies involving the systemic correction of protein insufficiency, even gaining access to immune-privileged organs through the elevation of serum levels of therapeutic protein. In this series of experiments, the use of a clinically relevant lentiviral vector to constitutively express GUSB in the donor cell population produced exceptional correction of disease *in vivo*, with no evidence of negative effects on the pluripotency or proliferative capacity of mesenchymal stem cells [13]. Considering the ease and efficiency of MSC transduction combined with their dispersal to multiple tissues following transplantation, human MSC have potential as a successful therapy option for MPSVII disease and other enzyme deficiencies.

5. Biosafety of genetically engineered human MSC

Numerous studies have now suggested that MSC have fewer complications regarding the insertion of virally delivered transgenes. Whereas HSC seem to maintain a highly quiescent pool of true stem cells [42–47] that are resistant to retroviral transduction, MSC seem to have no comparable metabolic barrier. Several studies have verified this finding, demonstrating that MSC can be efficiently and durably transduced without intensive labor and that this transgene expression is maintained throughout lineage differentiation and without compromising the proliferation rate or quality of progeny [48–51]. The clonal analysis of the resulting cell populations showed wide variation; however, some clones contained several thousand copies of transgene RNA per cell and were able to maintain this expression for up to 6 months post-transduction [50]. The examination of the starting cell population further showed that nearly 90% of all cells capable of producing CFU-F colonies were transduced using the standard procedures of the time [50]. In comparison to HSC transduction, this condition represented an astounding and intriguing finding for cellular therapy and genetic engineering.

However, a fear for MSC-based tissue therapy is that ectopic bone formation, or even tumor formation, could occur if the cells are not induced into the correct tissue at sites of damage. This fear is fostered by the fact that human and murine embryonic cells can form teratoma *in vivo* but should not apply to primary human cells that are subject to proper contact inhibition-mediated cessation of cell division. We have specifically searched, throughout two decades of using genetically engineered human MSC in highly sensitive transplant models, for any adverse events such as ectopic aberrant tissue differentiation or tumor formation occurring from human MSC *in vivo*. Our group's immune-deficient mouse studies with human marrow- and adipose-derived MSC are conducted under GLP (Good Laboratory Practice) conditions as mandated by the FDA, so they can be directly translational for MSC-based tissue repair therapies. We recommend similar methods of study and recordkeeping for other teams who plan to take MSC-based or other cell-based therapies into the clinic. All experimentation that could be used as supporting evidence for an Investigational New Drug (IND) application to the FDA must be conducted under GLP, with proper retention of source documents and experimental write-ups kept in a separate binder with tracking to the primary data storage site.

We have had many years of experience, at the level of GLP, in determining that tumors and ectopic unwanted tissues are not formed from human bone-marrow-derived MSC in immune-deficient mice when conducting careful biosafety studies for retroviral and lentiviral vector trials. Human hematopoietic stem cells and human mesenchymal stem cells carrying two different Moloney-based vectors were cotransplanted together into immune-deficient mice. A total of 481 mice were monitored for adverse events for 7–18 months post-transplantation. Following the cotransplantation of the engineered HSC/MSC inoculums, mice were assessed twice a day for signs of ill health, as defined by any of the following indicators: weight loss, hunching, lethargy, rapid breathing, skin discoloration or irregularities, bloating, hemi-paresis, visibly enlarged lymph nodes, or visible solid tumors under the skin. If any type of irregularity was observed, the mouse was immediately killed by 75% CO_2/25%O_2 narcosis, autopsied, and subjected to the full range of tissue and serum banking and biosafety analyses described below. If no signs of ill health were observed, the engraftment with transduced cells was allowed to

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continue until 7–18 months after transplantation. No evidence for insertional mutagenesis causing human leukemias or solid tumors in any of the mice was detected. No evidence for RCR in 117 serum samples analyzed by the vector rescue assay could be seen.

In addition, 149 mice were transplanted with human hematopoietic progenitor cells transduced with HIV-1-based lentiviral vectors and were followed for 2–6 months. No adverse events caused by the vectors could be observed, and none of the mice had detectable HIV p24 antigen in their serum. Our *in vivo* system therefore proves to be a valuable assay for potential risk assessment when retroviral or lentiviral vectors are considered to be used in human clinical gene therapy applications. We have published a manuscript that shows the safety of retroviral and lentiviral vectors (when they are not bearing a growth factor receptor gene, such as the common gamma chain), following up to 18 months of analyses of transduced human mesenchymal and hematopoietic cells cotransplanted into more than 600 mice with no immune system [14].

In summary, MSC-based cellular therapy, when combined with genetic engineering, can provide a safe and effective means by which to systemically produce factors that are needed by other cells in the organs of a recipient that has enzymatic or other defects. Numerous MSC-based therapies conducted by Osiris, Athersys, and other companies have demonstrated the safety of systemic infusion in phase I–II trials [5]. Biosafety data from our group and other researchers have further shown that the genetic engineering of MSC can provide a safe and effective cell-based therapy for different disorders where a single protein or enzyme is lacking. Genetically engineered MSC should be considered a cell-based therapy for some disorders, especially orphan diseases, when the risk to benefit ratio has been carefully considered.

Acknowledgements

This work was supported by the National Institutes of Health (NIH), the National Institutes of Diabetes and Digestive and Kidney Diseases [NIDDK #2R01DK61848 and 2R01DK53041 (JN)], and the National Heart, Lung and Blood Institute [NHLBI #RO1HL073256 (JN)]. The California Institute for regenerative Medicine (CIRM) provided funding for batches of human bone-marrow-derived MSC that were used in comparisons when in excess from directly funded studies [CIRM TR1-01257 (JN)]. Funding bodies supported salaries, equipment, mice and supplies needed for the collection and analysis of the data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.addr.2010.09.013.

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