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MESENCHYMAL STROMAL/STEM CELLS AS A GENE DELIVERY PLATFORM FOR PIGMENT EPITHELIUM-DERIVED FACTOR

Tutor:

Prof. Massimo Dominici

PhD Student:

Naomi Dsouza

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ABSTRACT

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein and is a non-inhibitory member of the serpin (serine protease inhibitor) superfamily of proteins. Its gene (*SERPINF1*) is located in humans on chromosome 17p13.3. PEDF interacts with components of the ECM. PEDF is found extracellularly in blood and interphotoreceptor matrix while its mRNA is widely expressed throughout the human body exhibiting multiple and varied biological activities. Most importantly, PEDF contains two peptide regions, 34-mer and 44-mer, for anti-angiogenic and neurotrophic signaling interactions, respectively. Little is known about the identification of PEDF-Rs however, two distinct receptors are proposed for PEDF: a 80 kDa PEDF putative receptor (PEDF-R^N) localized on motor neurons with high affinity to the 44-mer PEDF peptide involved in neurotrophic activity and a 60 kDa PEDF putative receptor (PEDF-R^A) localized on endothelial cells with high affinity to the 34-mer PEDF peptide involved in anti-angiogenesis. Moreover, PEDF-R encoded by PNPLA2 (also known as ATGL), laminin receptor, F1-ATP synthase and low-density lipoprotein receptor-related protein 6 (LRP6) also have been identified, though there are still missing pieces of the puzzle that are yet to be explored. PEDF has been described as a multifaceted protein with anti-angiogenic, anti-permeability, antiatherosclerosis, antitumorigenic, antioxidant, anti-inflammatory, antithrombotic, neurotrophic and neuroprotective properties. The anti-angiogenic activity of PEDF is selective because it is effective against newly forming vessels but spares existing ones, and the inhibitory effect of PEDF on vessel formation appears to be reversible in situations including tissue repair after injury. However, use of PEDF protein in clinic might be restricted because of its instability and short half-life. Thus, efforts should be made to develop effective and wide-applied strategy, and one potential solution for this is gene therapy.

Gene therapy–mediated expression is a practical and potentially cost-effective method for chronic delivery of anti-angiogenic agents. Mesenchymal stromal/stem cells (MSC) are multipotent, self-renewing cells that can be found in almost all postnatal organs and tissues and have been exploited as delivery vehicles to target antitumor agents to malignant cells. The ability of MSC to differentiate into a variety of cell types as well as their high ex vivo expansion potential makes them an attractive therapeutic tool for cell transplantation and tissue engineering. MSC can be readily transduced by viral vector systems to efficiently express a wide range of cytoplasmic, membrane-bound and secreted protein products. Keeping above literature in mind we first generated a retroviral vector coding for human PEDF gene (MIGR1-PEDF-GFP). Our vector was transiently transfected into 293T cells. Viral supernatant obtained from 293T cells was used to infect FLYRD-18 packaging cell line. Retroviral supernatant obtained from FLYRD-18 infected cells was then used to stably infect human AD-MSC and human BM-MSC samples obtained from multiple donors. Characterization by immunophenotype of AD-MSC and BM-MSC samples was performed by FACS. PEDF mRNA transcript levels in transduced cells was verified by real-time PCR after retro-transcription. Secreted levels of PEDF protein from genetically modified AD-MSC and BM-MSC were measured by ELISA. Proliferative rate of gene-modified AD-MSC and BM-MSC samples were determined by Ki-67 levels. Our gene-modified AD-MSC were also able to differentiate into different lineages. Furthermore, we induced our gene-modified BM-MSC to differentiate towards osteogenic lineage only and it was successful. We analyzed the expression of osteogenic related genes after induction of osteogenic differentiation in our AD-MSC and BM-MSC samples (wild-type and gene-modified). Collectively our data suggests that adult MSC can deliver PEDF thus representing a multipurpose therapeutic tool.

INTRODUCTION

MSC: Cellular Elements with Multimodal Properties

Mesenchymal Stromal/Stem Cells Biology

Mesenchymal stromal/stem cells (MSC) are multipotent progenitor cells, first identified by Friedenstein et al. who described a population of plastic adherent cells isolated from bone marrow with fibroblast-like morphology [1]. MSC are able to retain their robust proliferation as a result of their well-known clonogenic potential and self-renewal capacity [2].

In addition to a proliferative phenotype, MSC display differentiation capabilities that allow the generation of adipocytic, osteogenic, chondrogenic and myogenic lineages [3]. Additionally, MSC can act by secreting bioactive molecules capable of influencing normal and pathological tissue homeostasis [4]. These functions are exerted either ex vivo or *in vivo*. In the latter case, a specific homing mechanism further enhanced the potential of therapeutic approaches aimed to regenerate several damaged organs [5].

While MSC can be isolated from different tissues such as dental pulp [6], gingiva [7], umbilical cord blood (UCB) [8, 9], Wharton's jelly [10, 11], placenta [12] and are currently used for regenerative medicine, bone marrow and adipose tissue represent rich and more defined sources of MSC [13]. The MSC isolated from these different sources have similar properties and morphology, but differences were identified between UCB-MSC and bone marrow-derived (BM-MSC) and between adipose-derived MSC (AD-MSC) and BM-MSC. In particular, the UCB-MSC do not seem to have the ability to differentiate into adipogenic lineage however, they differentiate into osteogenic lineage within a short time frame as compared to BM-MSC. In addition, UCB-MSC does not show contact inhibition and proliferated faster than BM-MSC. No remarkable differences in morphology were observed between AD-MSC and BM-MSC, but AD-MSC had a faster doubling time and higher proliferative potential relative to BM-MSC [14].

In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) defined the minimal criteria that MSC should present: to be adherent in culture conditions, must express 95% of surface markers such as CD105 (transforming growth factor β receptor III or endoglin), CD73 (ecto-5'-nucleotidase) and CD90 (Thy-1), but should not express CD45 (pan-leukocyte marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD14 (macrophage marker), CD11b (monocyte marker), CD79a or CD19 (B-cell markers) [15]. Moreover, MSC express low levels of major histocompatibility complex (MHC) class I antigens and do not express MHC class II or costimulatory molecules such as CD40, CD80, CD86 [16]. On the contrary, *in vitro* expanded MSC are known to express a large number of adhesion molecules like CD90, CD44, CD29, CD105, CD73, CD166 (activated leukocyte adhesion molecules, ALCAM), CD106 (vascular cell adhesion molecule, VCAM-1) and Stro-1 [17–20]. Nevertheless, some of the above mentioned markers are not exclusive for MSC [21–23]. The origin of different tissues may be related to the differences in their expression. For example, CD49d is expressed on AD-MSC but not on BM-MSC; similarly CD106 is expressed on BM-MSC but not on AD-MSC. These immunophenotypical differences are also associated with the functionality of these cells within the tissue of origin. In fact, CD106 on BM-MSC has been functionally associated with hematopoiesis, and the lack of this antigen on AD-MSC is expected since it is related to the tissue which is not a hematopoietic tissue [24]. However, in all cases, the general rule of positive markers is CD73, CD90, and CD105 and the respective negatives as shown above, as a minimal criterion of expression for MSC in therapy.

Because of this variability in phenotypic analyses, a further way to identify supposed MSC populations, irrespective of source or culture conditions, is represented by differentiation potential. MSC are able to differentiate, when placed in appropriate *in vitro* or *in vivo*

environments, into different mesodermal cell lineages including osteoblasts, adipocytes and chondroblasts [15].

The classical method to differentiate MSC into osteoblasts *in vitro* involves the incubation of confluent monolayer of MSC with β-glycerophosphate, ascorbic acid-2-phosphate, dexamethasone and bone morphogenetic protein (BMP) for 2-3 weeks. The stages of osteoblast differentiation, namely proliferation, matrix maturation and mineralization, are paralleled by the expression of osteoblast phenotypic markers reflecting the stages of differentiation [25]. Whereas the proliferation stage demonstrates upregulation of genes associated with the cell cycle, the maturation phase demonstrates expression of the early osteogenic marker, alkaline phosphatase (ALP), and finally, mineralization is characterized by expression of late markers of differentiation such as osteocalcin and osteopontin [25]. β-glycerophosphate is an organic phosphate source which plays an important role in the mineralization process of primary cell cultures that can modulate ALP activity and influence the expression of osteocalcin [26]. Similarly, ascorbic acid plays a key role as a cofactor in the post-translational modification of collagen molecules and increases type I collagen production. It is well known that ascorbic acid stimulates and modulates the proliferation of various mesenchyme-derived cell types including osteoblasts, adipocytes, chondrocytes and odontoblasts ex vivo [27]. On the other hand, dexamethasone is a synthetic glucocorticoid, which has been shown to stimulate MSC proliferation and to support osteogenic lineage differentiation *in vitro*, together with β-glycerophosphate and ascorbic acid, in a concentration dependent manner [28]. Dexamethasone has been also identified to be able to increase the mRNA expression of runt related transcription factor 2 (Runx2) and ALP, stimulate the secretion of ALP ex vivo and also induce a decrease in the production of osteocalcin [29]. Lastly, BMP are members of the transforming growth factor β (TGF-β1-3) superfamily, among them, BMP-2 induces osteoblast differentiation and matrix

maturation *in vitro*. BMP-2 at 100 ng/mL significantly increased the expression of ALP, type alpha 1 (I) collagen, osteopontin, bone sialoprotein, osteocalcin and decorin in MSC [30]. Using these well-defined components in osteogenic differentiation culture conditions, MSC are able to upregulate ALP, Runx2, osteocalcin and osteopontin activity followed by a calcium deposition process within the extracellular matrix (ECM) as shown by the positive staining by Alizarin Red S or von Kossa [3].

To promote adipogenic differentiation, MSC cultures are incubated with dexamethasone, insulin, isobutyl methyl xanthine and indomethacin which activate the nuclear receptor and transcription factor, peroxisome proliferator-activated receptor-gamma (PPAR-γ), lipoprotein lipase (LPL) as well as fatty acid synthetase. These events leads to the production of lipid-rich vacuoles within cells identified by Oil Red O staining [3].

Finally chondrogenic differentiation takes place when MSC are grown under conditions that include a three-dimensional culture format, serum-free culture medium and the addition of BMP-6. In these conditions, the cell pellet develops a multilayered, matrix-rich morphology and histological analyses reveal a strong staining with Alcian blue, indicating an abundance of glycosaminoglycans within the ECM. The differentiated cells also produce type II collagen, which is typical of the articular cartilage [3].

Moreover, the differentiation towards other lineages originating from the mesoderm (skeletal and myocardial muscle cells) as well as non-mesoderm (neurons, hepatocytes or epithelial cells) remain questionable and may be strictly dependent on the origin of MSC [31–34]. In this context, it has been described that AD-MSC, but not BM-MSC, are also able to differentiate into endothelial cells [35], or cardiomyocytes [36]. The molecular and environmental mechanisms that control MSC differentiation are not fully understood, and no unique phenotype marker has yet been associated with predictable differentiation potential of MSC. There are currently several

hypotheses to explain the differentiation potential for MSC. For instance, Dennis and colleagues suggested that in MSC, there are storage genes that can express and adjust differentiation into various lineages when exposed to different conditions [37]. Phinney and Prockop proposed that MSC are equipped with motor proteins and a proteolytic arsenal that enables them to interact with and respond to signals from the ECM and differentiate into unique structures such as muscle, bone, cartilage, or other connective tissues [38].

Besides their differentiation potential, MSC possess numerous other characteristics including *in vitro* and *in vivo* immunoregulatory properties [39], trophic effects mediated by the wide range of growth factor and cytokines production [38] and the ability to migrate in inflammatory sites [40–43]. The broad range of *in vivo* effects of MSC have aroused relevant interest in several biomedicine fields such as immunology, regenerative medicine and, recently, its use has been observed in gene therapy approaches [44–47].

Bone Marrow: the Historical House of MSC

MSC can be isolated from different sources; the first was identified within the marrow. BM contains at least two main cell types: hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). BM-MSC may be a particularly important MSC population to consider for three reasons. First, their activity supports that of another distinct population of progenitor cells that give rise to cells of the blood and immune system: hematopoietic progenitors (reviewed in [48]). Second, BM-MSC could be used to encourage the repair of extramedullary tissues (reviewed in [49]). Third, BM-MSC may influence bone growth and remodeling. They form the osteoblasts that deposit osteoclacin in mineralized bone tissue, and through paracrine stimuli can regulate osteoclasts, which orchestrate osteocalcin release and activation through bone resorption [50, 51]. Unfortunately, the frequency of MSC in BM is low; MSC represent 0,001–0,01% of BM

mononuclear cells or lower [52]. Although the optimal dosage of MSC in therapeutic applications is still unclear and will be dependent upon the type of therapy, 1,0–2,0x10^6 MSC per kg body weight is generally used [52]. Direct collection of such a large number of MSC from BM is not practical. Therefore, it is necessary to expand isolated MSC *in vitro* to obtain a sufficient number for therapeutic approaches. Possible techniques to isolate MSC from BM materials include cell adherence based methods (such as density gradient centrifugation using Ficoll or PerColl) and cell-sorting methods, with the vast majority of previous clinical trials using the former method. The latter including fluorescence-activated cell sorting (FACS) and immune-magnetic bead cell sorting [53] has the advantage of collecting a more purified MSC population. However, they are hardly used in clinical trials because of the lack of specific simple surface markers for MSC, possible cellular damage, more expensive cost, and more demanding labor. Since several researchers have provided contrasting combinations of MSC markers there is an utmost need for BM-MSC characterization [48, 54, 55]. The confusion in the literature may be resolved if BM-MSC represent a heterogeneous population of progenitors at different stages of differentiation as BM-MSC may reside in three distinct niches within bone, namely, endosteal, stromal and perivascular niches, with different immunophenotypic features within each (reviewed in [56]). Conversely, some researchers have argued that MSC are a homogeneous population [57]. Those who argue that MSC are a homogeneous population have found CD271 (the low-affinity nerve growth factor receptor, a p75 neurotrophin receptor) to be the most consistent marker of these cells [57]. However, these authors also admit that levels of CD146 expression are not uniform across the population. Sacchetti and colleagues have shown that at least one subpopulation of CD146+ CD45- cells can be enriched from human BM-MSC preparations. This CD146+ population is capable of self-renewal and reconstitution of bone and hematopoiesis supporting stroma when transplanted into mice [58]. This is important because *in vivo* assay of cellular

activity is the most reliable means of BM-MSC characterization. CD146+ could therefore be one characteristic marker of BM-MSC or of a subpopulation of early BM-MSC progenitor cells. Other researchers have identified CD146 as a marker specific to BM-MSC compared to HSC, and have shown the *in vitro* chondrogenesis, osteogenesis and adipogenesis differentiation abilities of CD146+ BM-MSC [59]. Furthermore, an age-related decline in BM-MSC expressing CD146 has been reported [60]. The BM-MSC culture is composed of colony-forming units-fibroblastic (CFU-F)-a mix of tri-, bi-, and unipotent cells. The relative rate of these populations determines the potential of their growth, senescence and differentiation (in a multipotent manner) in the BM-MSC culture. Because of the heterogeneity of these cell populations, BM-MSC possess distinct differentiation potentials. This is likely due to the fact that BM-MSC has subpopulations of MSC in different stages of differentiation and the homogeneity of the population is dependent on the tissue of origin, isolation method and passage number. However, a myriad of studies show the capacity of MSC to differentiate in to adipose, tendon, muscle, cartilage, bone and nervous tissues [61]. In 1997, Komori et al. discovered that some marrow stromal cells express the master osteogenic transcription factor, Runx2 [62]. Interestingly, regardless of differentiation, the BM-MSC retain Runx2 expression, suggesting an ability to shift phenotype and re-differentiate into osteoblasts [63, 64].

Recent advance in basic and medical science and technologies has realized the employment of BM-MSC for a variety of therapeutic indications including regenerative therapies. One of the major hurdles for this development will be the establishment of optimized and standardized GMP-compliant protocols for isolation and expansion of MSC. It is now urgently important to solve this issue of the lack of conformity between MSC manufacturing protocols, which is considered as a potential threat for the further development of MSC-based therapy.

Fat: a Softer House for MSC

While BM has been recognized as the first source of MSC [65], adipose tissue represents an additional valid reservoir of mesenchymal progenitors [66]. The adipose tissue is an abundant and accessible source of stem cells, which possess the ability to differentiate into several cell types. The International Fat Applied Technology Society adopted the term "adipose tissue-derived stem cells" (AD-MSC) to identify the cell population isolated from the adipose tissue [67]. This population is characterized as plastic adherent and multipotent cells [66]. The adipose tissue is composed mainly of fat cells organized in lobules, which contain mature adipocytes in more than 90% of the tissue volume, and a fraction of the vascular stroma (SVF), where pre-adipocytes, fibroblasts, smooth muscle cells, endothelial cells, resident monocytes/macrophages, lymphocytes and AD-MSC are found [68, 69]. Adipose tissue can be obtained in relevant amounts under local anesthesia with a minimum of patient discomfort and can be easily processed to release large numbers of AD-MSC, therefore it represents a suitable tool for tissue regeneration and gene therapy approaches [24, 70, 71]. AD-MSC are usually isolated from adipose tissue by enzymatic digestion. The pellet obtained is then re-suspended in maintenance media, and plated in culture dishes or flasks. Usually, the yield of AD-MSC obtained from 1g of adipose tissue is $5x10^3$ stem cells, which is about 500-fold greater than the number of BM-MSC obtained from 1g of bone marrow [72]. The cells obtained after enzymatic digestion exhibit a fibroblast-like shape phenotype quite similar to the BM-MSC counterpart. This similarity is also confirmed by an overlap in both the immunophenotypical features and in the differentiation capacity [73–75]. Dealing with differentiation potentials, there are numerous examples from literature demonstrating the multipotency of AD-MSC *in vitro*. AD-MSC originate from a mesodermal tissue and are able to differentiate into adipogenic, osteogenic, chondrogenic lineages, moreover they differentiate also into skeletal and smooth muscle cells and into

cardiomyocytes [24, 36, 76, 77]. The expression of CD105, CD73 and CD90 proteins, and the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen-DR (HLA-DR) proteins have been provided as evidence for AD-MSC [78]. CD73 is considered as a characteristic marker of mesenchymal cells, and has been reported in addition to CD29, CD44 and SH3, which are also specific molecular markers characteristic of MSC. The expression of Stro-1 which is usually used as marker for bone marrow progenitor cells has also been found in AD-MSC. In contrast, the expression of hematopoietic lineage markers such as CD31, CD34, and CD45 was not detected in these cells through flow cytometry or immunofluorescence assays [78]. Alterations in the expression profile can be attributed to timing and passage number. After two or more successive passages in culture, the characteristics of adhesion molecules, surface receptors and enzymes, ECM and cytoskeleton proteins, as well as proteins associated with the phenotype of stromal cells can be altered. Nevertheless, despite differences in the cell isolation and culture procedures, the immunophenotype of AD-MSC is relatively consistent across diverse studies [79]. Successive AD-MSC passages may lead to higher expression levels of CD117 (c-kit), HLA-DR, as well as stem cell makers, such as CD34. At the same time, decreased levels of expression of stromal cell markers such as CD13, CD29 (β1 integrin), CD44 (hyaluronate), CD63, CD73, CD90, CD105 and CD166 have also been demonstrated. Additionally, the slight presence of CD106 has been reported in cultured AD-MSC [80]. A further study has shown that, after the third passage, the expression of OCT4, c-kit and CD34 in cultured AD-MSC is abolished, while Sca-1 expression has a significant enhancement. These cells have been identified as positive for CD29 and CD44, and negative for CD45 and CD31 expression [81]. Finally, in addition to the surface markers mentioned above, smooth muscle β-actin, platelet-derived growth factor (PDGF) receptor-β and neuro-glial proteoglycan 2 have also been identified in human AD-MSC [82]. Lastly, the expression of chemokine receptors,

such as, CCR1, CCR7, CXCR4, CXCR6 appears to be increased in AD-MSC compared to BM-MSC [83].

The Multimodal Properties into Clinic

The ability of MSC to preferentially migrate towards local and disseminated malignant disease, interact with different tissue environments in addition to their easy availability, non-immunogenic nature, relative ease of manipulation *in vitro* without requiring immortalization presents them as the most attractive candidates for cell based therapies in humans. MSC-based therapies have been evaluated for their safety and varying levels of effectiveness for treating various conditions including bone and cartilage repair, stroke, multiple sclerosis, diabetes, and kidney transplantation in other clinical trials [84]. The first clinical trial using culture-expanded MSC was carried out in 1995; in this study, 15 hematooncology patients received injections of autologous BM-MSC as part of a safety and feasibility study [85]. Human-marrow derived MSC autografts were one of the first successes in stem cell therapies as there is minimal chance of immune rejection due to their intrinsic immunomodulatory properties [39]. In addition, the most significant results on the immunosuppressive effects of MSC so far have been observed in the treatment of acute Graft-versus-host-disease (GVHD) after allogeneic stem cell transplantation. The first case of ex vivo expanded haploidentical MSC infusion in a patient with severe Grade IV GVHD of the gut and liver resulted in a striking improvement of the disease [86]. A Phase II study reported that 30 of 55 patients had a complete response and 9 patients showed improvement indicating that irrespective of the donor, MSC infusion might be an effective therapy for patients with steroid-resistant acute GVHD [87]. Since these studies were performed, several others have produced encouraging responses, both in acute and in chronic GVHD refractory to standard steroid treatment [88]. More recently, improved tissue engineering methods

have reduced the time required for graft generation from 3 months to 3 weeks allowing patients requiring more urgent transplants to be treated [89]. Moreover, these MSC transplantations do not typically result in teratoma formation when tested in clinical trials and are relatively safe compared to embryonic stem cells (ESC) and induced-pluripotent stem cells (iPSC) which readily form teratomas [90]. The clinical translation of UCB-MSC will be limited by their unreliable and often low isolation efficiency and requires allogeneic transfer. In contrast, allogeneic transfer is not necessary for adipose or BM-MSC, in which case an autograft can easily be harvested from any patient. Importantly, non-immortalized adult stem cells do not confer the same danger as immortalized adult stem cells and may be used without posing risk to the patient. In one such study, 41 patients who underwent BM-MSC transplant for joint repair were examined for tumor and infection symptoms for between 5 and 137 months, and no abnormalities were detected [91]. Another study of MSC transplantation for orthopedic therapy involving several hundred patients over a period of 1-2 years also argues that these transplantations are unlikely to increase the risk of carcinogenesis [92]. Other studies have also indicated that MSC transplantation is safe and has led to improved prognosis for orthopedic ailments [93, 94].

MSC were originally evaluated for their ability to repair skeletal defects first in experimental animal models [49] and subsequently in human patients affected with osteogenesis imperfecta (OI), a bone genetic disease generally caused by mutations in genes encoding for type I collagen. After first demonstration of the potency of MSC to differentiate into functional osteoblasts in a mouse model of OI, MSC were used in children with severe type of OI with a transient but substantial effect [95]. Recently, Cowan and colleagues used AD-MSC to successfully heal calvarial defects in mice, representing the first solid report of bone repair using AD-MSC [96]. In human, the first case of autologous AD-MSC use for bone repair is reported for the treatment of

calvarial defect in a 7-year-old girl [97], in this study a porous sheet seeded with pulverized bone and freshly isolated AD-MSC were implanted into the defect. The ossification process was visualized by computed tomography scanning and it was sufficient to allow the discontinuation of the patient's protective helmet.

The safety of MSC therapies for myocardial infarction (MI) has also been accessed, and patients showed improved cardiovascular prognosis [98]. In addition, Mohyeddin-Bonab and his team reported that on injection of autologous BM-MSC in 8 patients with heart failure after MI; 3 patients received intracoronary injections and the remaining 5 received myocardial injections during coronary bypass surgery [99]. Patients experienced significant improvement of left ventricular (LV) ejection fraction as compared to controls. Currently, ongoing clinical trials using AD-MSC for cardiovascular treatment has been reported. In a particular study, freshly isolated AD-MSC were delivered through intracoronary infusion within 36 hours following the onset of heart attack. Results from 14 patients showed that AD-MSC were able to improve cardiac function of ischemia patients. At 6 months, an improvement of LV ejection fraction and reduction in infarct size in the AD-MSC treated group [100] was noted. Now, a phase II/III ADVANCE trial has been initiated to evaluate their efficacy (https://clinicaltrials.gov/ct2/show/NCT01216995).

On a different aspect, Agorogiannis et al. presented a case report in which patient with post-traumatic persistent sterile corneal epithelial defect were treated with topical application of autologous AD-MSC [101]. Corneal epithelial healing progression was started 11 days after topical application of autologous AD-MSC. One month later, a complete corneal epithelial healing was observed [101]. AD-MSC were also used to heal chronic fistulas in Crohn's disease [102, 103]. This disease is an inflammatory bowel disorder characterized by bloody stools, diarrhea, weight loss and autoimmune-related symptoms. In a phase I trial with patients with

fistulas unresponsive to standard treatment, cultured AD-MSC were directly injected into the rectal mucosa, and 75% of cases healed completely. In a phase IIb trial, the proportion of patients who achieved fistula healing was significantly higher with AD-MSC than with fibrin glue alone [104]. There are also a number of ongoing clinical trials which are utilizing MSC for cancer therapy, however most of these trials do not use engineered MSC and the results of any adverse effect from such trials is still awaited [45].

Emerging data demonstrate that after engraftment, MSC can contribute to tissue repair by secreting a number of trophic molecules that include soluble ECM glycoproteins (collagen types I and II, osteopontin), cytokines (transforming growth factor [TGF]-β, IL-10, IL-6), and growth factors (vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), keratinocyte growth factor) [105]. These trophic molecules promote cell–cell connections [106]. It has been observed that these trophic molecules can not only reduce inflammation, apoptosis, and fibrosis of damaged tissues, but also stimulate tissue cell regeneration. Although there is evidence that MSC and certain tissue cells such as cardiomyocytes can interact with one another via small diameter nanotubes, the underlying mechanism of cell–cell connection and its possible roles during tissue regeneration remains to be further investigated [106, 107]. From these studies it is clear that adult stem cells derived from patient tissues may not only be a source of cells for autologous regenerative therapies but also in studying mechanisms of disease, in drug screening and toxicology tests that are crucial in new drug development. Moreover, stem cell industry is witnessing continuous advancements globally and hence, many products are underway. The widespread availability of stem cell therapies will not only help make the treatment affordable in the coming years but also pave the way for personalized medicine.

PEDF: a Swiss-knife Protein with still Undefined Mechanisms of Action

The Serpins Family

Serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors [108]. They are single chain (glyco-) proteins comprising of about 400 amino acid residues, most of which are synthesized in liver and abundant in plasma [109]. Serpins are involved in a number of fundamental biological processes such as blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation and tumor suppression and are expressed in a cell-specific manner [110]. Defects in serpins can have pathological consequences and contribute to diseases such as thrombosis, emphysema, angioedema and cancer [111]. Serpins are of structural interest because of their remarkable ligand-binding features and exquisite kinetically controlled folding properties that are critical for inhibition of target proteinases [112]. The serpins family encodes two groups of proteins. The first group comprises the predominant family of protease inhibitors in mammals and regulates cascades such as inflammation, blood coagulation and ECM remodeling [110]. The second group represents a substantial number of serpins that are not thought to be inhibitors of specific proteases but rather perform diverse functions such as hormone transporters, molecular chaperones or tumor suppressors [113]. Non-inhibitory serpins include many secreted, cytoplasmic and nuclear proteins whose structural mechanisms of action are less well understood [112]. Among such non-inhibitory serpins we will focus on Pigment epithelium-derived factor.

The Pigment Epithelium-Derived Factor (PEDF)

PEDF was first discovered by Tombran-Tink and Johnson in 1987 as a product secreted by cultured pigment epithelial cells from fetal human retina [114]. The sequence of human, bovine

and mouse PEDF cDNA revealed that PEDF has structural and sequence homology to members of the SERPIN family of proteinase inhibitors and contains a reactive centre loop (RCL) that is characteristic of this family [115]. RCL is a proteinase recognition site and a critical component of the function of serpins but the function of RCL structure within PEDF is still unknown [116]. PEDF is a non-inhibitory member of the serpin (serine protease inhibitor) superfamily of proteins [117] i.e. it does not exhibit inhibitory activity against proteinases, rather can serve as their substrate. PEDF does not undergo the serpin conformational change that affects inhibitory serpins on interacting with target proteases [118].

The human PEDF gene (*SERPINF1*) is located on syntenic region (B4) on mouse chromosome 11 and in humans it is located on chromosome 17p13.3, a region containing a cluster of cancer-related genes [115]. PEDF encodes a 418 amino acid protein [119] and is a stable soluble 46.3 kDa endogenously secreted glycoprotein. PEDF contains a single carbohydrate side chain that increases its apparent molecular weight to 50 kDa. The human PEDF gene contains 8 exons and 7 introns and is around 16 kb (14.8 kb in humans), and the mRNA transcript is around 1.5 kb [120]. The crystal structure for human recombinant PEDF has been solved and is at a resolution of 2.85Å [121]. It has an asymmetrical charge distribution, with a high density of basic residues concentrated on one side and of acidic residues on the opposite side (Figure 1).

PEDF interacts with components of the ECM, such as glycosaminoglycans [122, 123] and collagens *in vitro* [109]. It is therefore speculated that the interaction of PEDF with different ECM components could allow for different PEDF activity [124]. Negatively charged acidic PEDF binds to collagen, lacks neurotrophic activity, and may confer anti-angiogenic properties whereas positively charged basic PEDF binds to heparin [125]. Analyses using residue-specific chemical modification and site-directed mutagenesis, revealed that the acidic amino acid residues on PEDF (Asp256, Asp258, and Asp300) are critical to collagen binding, and three clustered

basic amino acid residues (Lys146, Lys147, and Arg149) are necessary for heparin binding [126, 127] whereas Lys189, Lys191, Arg194 and Lys197 form a motif that is critical for hyaluronan binding [128]. In addition, the phosphorylation state of PEDF on the residues Ser24, Ser114, and Ser227 was shown to impact its biological function as neurotrophic and/or anti-angiogenic factor [129] suggesting that PEDF bioactivities could be regulated at different levels.

PEDF: Sources & Functions

PEDF mRNA is widely expressed throughout the human body such as in the eye, brain, spinal cord, pancreas, prostate, lung, liver, heart, bone and adipose tissue, which exhibits multiple and varied biological activities [130, 131]. Immunoblotting and immunohistochemistry analyses have shown that the PEDF protein localizes to the interphotoreceptor matrix in bovine, human, mouse, and rat eyes and cells of the retinal pigmented epithelium (RPE) can secrete soluble PEDF into the media [132]. The concentration of PEDF in the vitreous in several species has been estimated to be between 1-2 μg/ml or 20–40 nM [133]. These levels are about 10-fold higher than those found in the aqueous, but almost 10-fold lower than those in the interphotoreceptor matrix. PEDF is also reportedly present in human plasma at a concentration of around 5 μg/mL [134]. Due to the fast secretion of PEDF, it has been difficult to localize the protein in certain conditions [109]. PEDF is one of the most abundant circulating proteins in humans, accounting for 0,02% of total circulating proteins [135]. The predominant sources of circulating PEDF are thought to be liver and adipose tissue [136, 137]. Hepatocytes secrete abundant PEDF [138], hepatic PEDF expression is 6-fold higher than in retina [139] and serum PEDF is decreased in humans with cirrhosis [140]. However, it has been noted that in adipose tissue PEDF levels are down-regulated during differentiation process to mature adipocytes [141].

Tombran-Tink et al. observed that media conditioned by retinal pigment epithelial cells from human retina induced Y79 retinoblastoma tumor cell differentiation into a non-proliferating type with a neuronal morphology that exhibited an increase in neurite outgrowth [142]. The protein was isolated from the conditioned media and defined as PEDF. Further studies revealed that addition of PEDF at nanomolar concentrations to the media of human retinoblastoma Y79 and Weri cells induces a neuronal phenotype, which is accompanied by the expression of neuronal-specific markers such as enolase and 200 kDa neurofilament [115, 143]. PEDF also exhibits neurotrophic activities on primary cultures of rat cerebellar granule (CG) neurons, such as neuronal survival [144] and protection against death by glutamate neurotoxicity [145] and by apoptosis [146]. In addition, it delays the death of photoreceptors in mouse models of inherited retinal degenerations (retinitis pigmentosa) [147]. This marked the role of PEDF as a neurotrophic factor, soon after; PEDF was defined as the most potent inhibitor for angiogenesis among other well-characterized anti-angiogenic factors; it is twice as potent as angiostatin and more than seven times as potent as endostatin [148]. PEDF blocks proliferation, migration, tube formation and induces apoptosis in endothelial cells activated by various angiogenic inducers, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and acidic fibroblast growth factor (aFGF) [148]. The anti-angiogenic activity of PEDF is selective because it is effective against newly forming vessels but spares existing ones, and the inhibitory effect of PEDF on vessel formation appears to be reversible when transient and regulated angiogenesis occurs in situations including tissue repair after injury [149]. In addition to being anti-angiogenic, neurotrophic and neuroprotective in nature PEDF has been described as a multifaceted protein with anti-permeability, antiatherosclerosis, antitumorigenic, antioxidant, anti-inflammatory and antithrombotic properties [150]. These studies may suggest that PEDF uses distinct intracellular signaling mechanisms to exert different

functions through different molecular domains. The effects of PEDF are dependent on target cell age and type (e.g., presence/absence of receptors, cytoplasmic signals, glycosaminoglycan production, pH). PEDF is also called EPC-1 (early population doubling level cDNA-1), reflecting its upregulation during cell cycle arrest (G0) in young but not senescent cells *in vitro*. The PEDF mRNA was induced more than 100-fold when cells were arrested in the G0 state [151]. It exerts a paracrine effect and reduces the number of cells entering the S-phase [152]. PEDF promotes cellular differentiation and suppresses cell-cycle progression when cells become confluent [153] to allow some cells to enter differentiation programs. Thus, PEDF is considered to be a marker for cell senescence [154].

Recently, Gao et al. demonstrated that PEDF and its shorter 44-mer peptide protected cardiomyocytes against hypoxia-induced apoptosis and necroptosis via an anti-oxidative mechanism [155]. In bone, PEDF regulates neoangiogenesis to underlie the physiological processes of bone formation, growth, and remodeling [156]. PEDF was found to be expressed in the zones of active bone formation and its role in cell differentiation as well as the maintenance of high growth rates in the mesenchymal cell layer was determined by Lord et al. using a deer antler model [157].

Moreover, PEDF is a candidate tumor suppressor and inhibits tumor cell growth promoting tumor cell differentiation [158]. PEDF is proposed to be a tumor suppressor in a variety of tumors including glioma, prostate, pancreas, breast, and lung cancers; whereas in human breast and lung cancers, expression of PEDF is reduced and linked to enhancement of tumor growth, angiogenesis, and poorer clinical outcome [159].

PEDF seems also associated with adiposity, type 2 diabetes, and the metabolic syndrome. It has been shown to be an adipokine (adipocyte-secreted protein) that induces insulin resistance and plays a role in glucose metabolism [160]. The role of PEDF in various pathological conditions,

including chronic inflammatory disease, cardiovascular disease, angiogenic eye disease, diabetic complications and cancer have been investigated and reported in literature [161]. Thus, PEDF appears to be an important endogenous protein with wide pleotropic effects on cell growth and function and can be harnessed as a recombinant factor in different therapeutical areas.

The Unresolved Mystery of PEDF Receptors

PEDF is a growth factor and its actions are dependent on interactions with cell surface receptors and triggers intracellular signaling [131, 162]. A long-standing challenge has been to understand how PEDF acts on different cell types and its fundamental transmembrane mechanisms. Uncovering the transmembrane pathways of PEDF would lead to a better understanding of its fundamental mechanisms and the development of new therapeutic strategies. However, little is known about the identification of PEDF-R until recent studies have initiated a search for cell-surface proteins with a high affinity for PEDF. Two distinct receptors are proposed for PEDF: a 80 kDa PEDF putative receptor (PEDF-R^N) localized on motor neurons with a high affinity to the 44-mer PEDF peptide involved in neurotrophic activity and a 60 kDa PEDF putative receptor (PEDF-R^A) localized on endothelial cells with a high affinity to the 34-mer PEDF peptide involved in anti-angiogenesis [163]. Filleur et al. showed *in vivo* that overexpression of the 34-mer in PC-3 prostate adenocarcinoma cell lines resulted in decreased tumor microvessel density and increased apoptosis, whereas the 44-mer lacked anti-angiogenic effects but induced neuroendocrine differentiation [164]. Hence, the differential expression of PEDF-R^N and PEDF-R^A on endothelial and tumor cells could contribute to its distinct actions. The first identified receptor, PEDF-R^N, was found in the retina and is a novel phospholipase and triglyceride lipase involved in triglyceride metabolism [131]. It is also termed PNPLA2 (patatin-like phospholipase domain containing protein 2), adipose triglyceride lipase (ATGL) [162], a highly conserved

triacylglycerol lipase that is critical for the maintenance of lipid and glucose homeostasis [165]. However, the signaling pathway is still under investigation. PEDF-R is a transmembrane protein with 4 transmembrane, 2 extracellular and 3 intracellular domains, the N- and C-termini of the molecule are exposed into the intracellular environment [162]. Human PEDF-R was mapped to locus 11p15.5 on chromosome 11 and consists of 10 exons and 9 introns. Inspection of the PEDF-R cDNA sequence revealed mRNA transcript of 2122 bases long with a coding capacity for a polypeptide of 504 amino acids (molecular mass of 55.315 kDa) and 4 consensus N-glycosylation sites. When expressed in eukaryotic systems, the receptor has a mass of approximately 81 kDa, which is similar to the mass of PEDF-binding proteins on its cell's surface [166]. It has specific and high binding affinity for PEDF through its extracellular loop. The receptor is mainly found in retinal pigment epithelium cells, the inner segments of photoreceptors and neuronal cells of the retina, though it is not detected in the outer segments of rods. PEDF-R transcript was also found to be highly expressed in adipose tissue and less extensively in various organs such as prostate, testes, uterus, thymus, skin and skeletal muscle tissue. The second receptor for PEDF, PEDF-R^{A}, is also a receptor for laminin [167]. On binding of the 25-mer PEDF region (25-mer peptide lies within the 34-mer region of PEDF) to the laminin receptor, endothelial cell apoptosis is initiated while angiogenesis, migration, tumor cell adhesion and proliferation are inhibited. Recently, Notari et al. identified that PEDF and angiostatin could interact and inhibit endothelial and tumor cells by surface F1-ATP synthase. The interaction of PEDF and angiostatin occurs at the same location on the F1-ATP synthase and prevents the formation of ATP from ADP and inorganic phosphate by the enzyme. ATP and ADP have receptors on cell surfaces and would constitute mediators of PEDF. A change in ATP levels could affect cell viability and proliferation negatively. Given that the enzyme is also a proton pump, it is proposed that these anti-angiogenic factors could prevent the exit of protons from

cells resulting in a decrease of intracellular pH leading to cell death. Agents that inhibit exclusively the cell surface F1-ATP synthase, such as PEDF, could be used as agents to prevent endothelial and tumor viability. This ectopic enzyme is being considered as another receptor for PEDF [168]. Despite the discovery of PEDF-Rs there are still missing pieces of the puzzle that are yet to be explored, especially the molecular pathways involving PEDF and PEDF binding to its receptors.

PEDF Safety, Half-life and Clinic

Among the different functions, PEDF specifically and potently suppresses pathogenic neovessel growth without harming mature vessels [112, 148, 169]. Another prominent advantage of using angioinhibitors is that being physiologically present in the body and in plasma it would be hard to imagine that it would exhibit cytotoxicity. Additionally, no ADME/Tox studies have been performed on the protein or its shorter peptides. However, its effects on physiologic neovasculature, such as that in the menstrual cycle and wound healing, will nevertheless need addressing. More, in osteosarcoma patients, any counterproductive effects on proper bone growth as well as bone formation and repair that require vascularization will also demand attention.

From literature data, PEDF is not expected to activate drug-resistance genes, and thus offers the potential for effective long-term anti-angiogenic therapy [169]. When applied sub-conjunctivally into rats less than 1% of the PEDF protein reached the choroid and its levels decreased to less than 0,1% after 24 hours thereby restricting its application in clinic because of its instability and short half-life [170]. Based on these evidences, efforts have been made to develop effective and wide-applied strategy by means of gene therapy.

PEDF: a Swiss-knife for Angiogenesis, Cancer and Osteogenesis

PEDF Molecular Pathways Against Angiogenesis

Angiogenesis involves formation of new blood vessels from pre-existing microvascular networks and is required for a variety of physiological processes such as reproduction, development, wound repair and tissue regeneration. Angiogenesis is a multistep process consisting of endothelial cell proliferation, migration, basement membrane degeneration (including collagen, laminin, proteoglycans, and glycosaminoglycans) and new lumen formation. These cellular responses of endothelial cells are tightly regulated by a balance between pro-angiogenic and anti-angiogenic factors [171]. Among which VEGF is characterized as the best pro-angiogenic factor and PEDF being the most potent anti-angiogenic factor. A shift in the balance towards pro-angiogenic factors leads to neovascularization, a process intrinsic to pathological conditions ranging from chronic inflammatory diseases to cancer [172]. Pro-angiogenic inducers include VEGF, basic fibroblast growth factor (bFGF) and IL-8 [173] that promote endothelial cell survival and are expressed in most tumors [174]. Apart from promoting migration, proliferation, and permeability of endothelial cells, VEGF also prevents apoptosis of endothelial cells even in conditions of serum starvation [175]. VEGF stimulates Akt, Bcl-2, A1, XIAP, survivin (all of which oppose apoptosis) and, most notably, cellular FLICE-like inhibitory protein (c-FLIP), an inhibitor of caspase-8 and of Fas-induced apoptosis [176]. The roles of anti-angiogenic agents are important when considering therapeutic strategies for neovascular diseases. These agents can affect various aspects of the angiogenic process, including vascular atrophy, endothelial migration and proliferation, and the three-dimensional restructuring of blood vessels. Signaling events of PEDF in endothelial cells is well described in figure 2. During neovascularization, VEGF binds to a homodimerized VEGF receptor (VEGFR), becomes phosphorylated and

activated. PEDF can increase γ-secretase-mediated cleavage of VEGFR1 and VEGFR2 at the transmembrane region to generate an intracellular domain fragment [177, 178]. At the same time, PEDF can inhibit VEGF-induced phosphorylation and activation of VEGFR1 [179]. Thus, PEDF inhibits VEGF-driven angiogenesis and permeability through the regulated intracellular proteolysis of VEGFR. PEDF can also activate the p38 mitogen-activated protein kinases (MAPK) pathway to inhibit endothelial cell migration [180]. It can also activate peroxisome proliferator-activated receptor-γ (PPARγ) through cytosolic phospholipase A2α (PLA2α) to induce the expression of *TP53*, which encodes the pro-apoptotic protein p53 [181]. At the same time, endothelial activators such as VEGF and FGF2 stimulate and expose CD95 (also known as Fas) on the endothelial plasma membrane. PEDF can sequentially activate MEK5 (which is a MAPK kinase), extracellular signal-regulated kinase (ERK5), PPARγ and nuclear factor-κB (NF-κB), which induces the expression of the pro-apoptotic gene CD95 ligand (CD95L or FasL), the protein product of which translocates to the plasma membrane. The resulting CD95L–CD95 complex induces the binding and activation of caspase-8 that under certain conditions triggers the cell death cascade [176, 182]. At the same time, NF-κB activation has a negative impact on c-FLIP expression, which decreases the capacity of c-FLIP to inhibit caspase-8. Conversely, PEDF triggers JUN N-terminal kinase (JNK)-mediated phosphorylation of nuclear factor of activated T-cells, cytoplasmic 2 (NFATc2) and sequesters it in the cytoplasm, thus blocking c-FLIP expression. In this manner, PEDF causes apoptosis of activated endothelial cells. Moreover, an *in vivo* study showed that in PEDF knockout mice, absence of PEDF is unlikely to be essential for viability of PEDF-deficient mice as they appear to be alive and healthy however, retina, kidney, prostate and pancreas developed hyperplasia with hypervascularization [183, 184]. These results confirmed that PEDF regulates processes associated with angiogenesis, such as, for normal organ development.

PEDF Therapeutic Strategies in Cancer

Cancer is a multistage process, and involves several molecular alterations such as the loss of tumor suppressor genes and gain of dominant oncogenes in the malignant cells; enhanced angiogenesis in the tumor environment; and additional immunological defects leading to the inability of the immune system to destroy the cancer cells [185]. Despite significant advances in surgical resection and neo/adjuvant chemotherapy and radiotherapy, there are a significant number of tumors that are unresponsive to aggressive treatment. Anticancer strategy in which the endothelial cells supporting tumor growth are targeted is appealing because the endothelial cells themselves are normal cells with a low intrinsic mutation rate and therefore are unlikely to acquire a drug resistant phenotype [186]. Tumor angiogenesis starts with cancerous tumor cells releasing molecules such as VEGF and FGF, which promote endothelial cell proliferation, migration and eventually capillary tube formation. The use of anti-angiogenic therapy is specific to the rapidly growing vascular endothelial cells (VEC) in tumors, without any effect on slow growing VEC and other normal cells. Thus, anti-angiogenic therapy should be an excellent strategy to suppress tumor growth and metastasis. Anti-angiogenic compounds such as blockers of VEGF activity (bevacizumab and avastin) and VEGF-receptor-kinase inhibitors (SU5415, PTK787 and ZD6474), all of which have shown promise in inhibiting tumor angiogenesis [187, 188] have been used in clinical trials. The finding that PEDF is a potent angiogenesis inhibitor has become the basis for studying the role of PEDF in tumors that are highly resistant to chemotherapy. PEDF inhibits the effects of several inducers of angiogenesis on endothelial cells including VEGF, bFGF, IL-8, lysophosphatidic acid and PDGF [148]. Researchers have performed a number of studies and have demonstrated that there is an opposite relation between PEDF levels, grade and metastatic potential of prostate tumors, pancreatic adenocarcinoma, melanoma, ovarian, osteosarcoma, glioma, hepatocellular carcinoma and Wilm's tumors [125].

Decreased intratumoral expression of PEDF is associated with a higher microvessel density, a more metastatic phenotype and poorer clinical outcome. Thus, measuring PEDF's concentration within the tissue or fluid of a cancer patient compared to healthy individuals may determine whether the tumor is on an early or advanced stage of tumorigenesis [163] though this remains a hypothesis at present. The mechanisms through which PEDF exerts its antitumor activity seems to be multiple, with studies showing widespread activities against tumor proliferation, cellular invasion, migration, differentiation and tumor angiogenesis. PEDF may possess both indirect and direct antitumor effects [130]. Indirectly, reduction in tumor growth is achieved through the anti-angiogenic action of PEDF, in which the selective targeting of newly formed vasculature without harming the existing blood vessels is of a particular importance [189]. PEDF also exerts a direct antitumor effect, possibly by inducing either antiproliferative or prodifferentiation activities toward cancer cells [190, 191]. PEDF's ability to suppress tumor cell proliferation has been shown to occur at two levels, through the induction of tumor cell apoptosis [191] and through the regulation of the cell cycle, leading to a decrease in the entry of cells into the S-phase [153]. The sum of PEDF's indirect and direct effects poses it as an emerging antitumor agent that may be able to target tumor cells without the attendant morbidity of conventional chemotherapy. Introducing exogenous full-length or fragments of PEDF enables targeting endothelial cells by inhibiting their proliferation and migration into the bloodstream through apoptosis of endothelial cells recruited by the tumor [163]. This method does not only prevent the expansion of blood vessels to the tumor but also inhibits tumor growth by restricting the tumor's supply of nutrients and oxygen. For instance, the treatment of exogenous full-length PEDF inhibits VEGF expression in cells and in mice, and administration or overexpression of PEDF leads to the inhibition of osteosarcoma tumor growth, which also disables metastasis to the lungs [192]. The same result was also evident when PEDF fragments, StVOrth-2 and -3, were given to mice with

orthotopic osteosarcoma. Furthermore, PEDF fragments StVOrth-3 and -4 were found to repress VEGF expression [193].

Another facet that PEDF may exhibit antitumor activity is in its ability to promote tumor cell differentiation. Crawford et al. demonstrated that Schwann cells, which are often mixed within the tumor-cell population, expressed high levels of PEDF and could induce tumor-cell differentiation *in vitro*, furthermore, in primitive neuroblastomas that were grown subcutaneously in athymic mice the intratumoral injection of recombinant PEDF resulted in tumor cell differentiation evidenced by less malignant appearing cells histologically and strong immunohistochemical staining for neurofilament, a marker for neural cell differentiation [158]. Filleur and colleagues showed in prostate cancer that PEDF exerts its anti-angiogenic and cell differentiation ability through two functional epitopes. They showed that a 44-mer peptide induced a neuroendocrine phenotype from prostate epithelium, which was manifested by dendrite-like processes, increased neuron specific markers, and secretion of neuropeptides [164]. Furthermore, when osteosarcoma cell lines were exposed to recombinant PEDF, there was a significant increase in mineralized nodule formation, further highlighting the role of PEDF in osteoblastic differentiation. Although the process through which this occurs is unknown, it is believed that the ability of PEDF to encourage cell differentiation towards a more mature phenotype, may contribute to its overall capacity to slow tumor growth kinetics and decrease metastatic spread [192]. However, anti-angiogenic therapy is cytostatic and hence requires constant therapeutic levels of anti-angiogenic factors *in vivo* to achieve its therapeutic effect, therefore recombinant proteins are limited for widespread clinical use [194]. Due to the need of high therapeutic doses of recombinant proteins the resultant yield rates of recombinant proteins may be low due to their denaturation during the purification process. Furthermore, owing to the short half-life of protein *in vivo*, maintaining therapeutically effective serum levels needs a

frequent dosing regimen and high doses of expensive purified recombinant proteins. The application of PEDF protein in clinic might be restricted because of its instability and short half-life [195]. Thus, efforts should be made to develop effective and wide-applied strategy, and one potential solution for this is gene therapy.

Unexplored PEDF Roles in Skeletal Biology: the recessive Osteogenesis Imperfecta (OI)

During the physiological process of endochondral ossification, the microenvironment of the growth plate changes from an angiostatic to an angiogenic one. This process is coordinated by a complex balance between specific pro-angiogenic factors (VEGF) and anti-angiogenic factors (PEDF) expressed at various levels of the growth plate. It culminates in the vascular invasion of the lowermost hypertrophic chondrocytes that is essential for endochondral ossification. A report on expression pattern of PEDF in developing mouse hind limbs from newborn through maturation, demonstrated that the protein is expressed in chondrocytes within the resting zone, proliferative and upper hypertrophic zones of the epiphyseal growth plate [156]. The pattern of expression was reported to be consistent throughout the developmental stages of the mouse. In addition, immunohistochemical analyses of human neonatal growth plates have shown high expression of VEGF in chondrocytes of the lower hypertrophic and mineralized zones, with no expression in the resting and upper proliferative chondrocytes [196, 197]. The expression of these pro-angiogenic factors by hypertrophic chondrocytes stimulates blood vessel invasion from the perichondral vascular network into the growth plate. These new vessels provide a conduit for the migration of many cells including osteoblasts and osteoclasts progenitor cells, which degrade the mineralized cartilaginous matrix and replace it with bone [198]. Thus, the close association of PEDF with VEGF, both temporally and spatially, at the sites of bone lesions and bone remodeling, suggest that these antagonistic factors play a very central role in such biological

processes. Quan et al. first investigated the expression of PEDF in regions of active bone formation [199] and Tombrank-Tink et al. confirmed PEDF expression in regions of active bone formation wherein PEDF expression increased from earliest ages right through maturity, most notably in the developing bone matrix [200].

Osteogenesis imperfecta (OI) is a heterogeneous heritable disorder of connective tissue characterized by reduced bone mass, bone fragility, and short stature. The majority of OI cases are due to dominant defects in either of the type I collagen genes, COL1A1 and COL1A2 [201] . The current standard therapy for moderate and severe forms of OI is cyclic intravenous application of bisphosphonates [202]. Bisphosphonate treatment, ideally starting in early childhood, increases bone mineral density. There is evidence that it also reduces fracture rates, chronic bone pain and immobility in OI patients [203]. Among the different OI variants, OI type VI is a moderate to severe, recessive form of the disease in which patients are typically present with fractures during infancy [204], malformed bones, and frequently have limited mobility by the time they reach adolescence. OI type VI is distinct from other types of OI in that the afflicted subjects display an osteomalacia-like phenotype characterized by thickened osteoid and delayed mineralization. A distinct 'fish-scale' lamellar appearance of the bone has been noted in bone biopsies [204]. This skeletal phenotype is still unclear and the severity of the disease requires a better understanding of the pathophysiology to then establish proper therapies. In addition, bisphosphonates are generally less effective in treating OI type VI than other subtypes of OI [205]. Moreover data from PEDF knockout mice demonstrated that the mice exhibited clinical characteristics of human OI type VI confirming that an inactivating mutation in the *SERPINF1* gene which encodes PEDF leads to the development of OI type VI [206]. Until now, 17 unique *SERPINF1* mutations (http://www.le.ac.uk/ge/collagen/) have been found in about 30 individuals with OI type VI [207]. In this OI variant, extremely low or undetectable levels of circulating

PEDF were found [208]. In addition, recent data suggest that the lack of PEDF impairs a proper osteoblast–osteocyte transition and consequently affects the early steps of mineralization, downstream collagen assembly making OI type VI different from “classical” OI despite the typical hypermineralization of the bone matrix [209]. Thus, it is possible to devise a treatment of OI type VI that is based on PEDF replacement treatment. However, before such a treatment can be devised, the differential roles of circulating PEDF and locally produced PEDF on bone metabolism need to be determined.

AIM OF THE STUDY

Pigment epithelium-derived factor (PEDF) is a non-inhibitory serpin widely expressed throughout the human body exhibiting multiple and varied biological activities. PEDF has been described as a multifaceted protein with anti-angiogenic, antitumor and neuroprotective properties. PEDF therapeutic role against angiogenesis, bone diseases and cancer has been briefly reviewed in the text. However, use of PEDF protein in clinic might be restricted because of its instability and short half-life. To overcome this limitation we propose a gene therapy strategy. Mesenchymal stromal/stem cells (MSC) are multipotent progenitor cells characterized by extensive self-renewal capacity and ability to differentiate into multiple lineages. MSC can be readily transduced by viral vector systems to efficiently express a wide range of cytoplasmic, membrane-bound and secreted protein products. Implementing MSC as delivery platform to combat malignant and non-malignant diseases has been described in literature. MSC can be isolated from different tissues and are currently being employed in several regenerative medicine applications. Among them, bone marrow (BM) and adipose (AD) tissue represent the richest and more defined sources of MSC. Thus, adipose-derived MSC (AD-MSC) and bone marrow-derived MSC (BM-MSC) could represent a powerful tool for cellular therapy and gene transfer applications. Based on this hypothesis, we genetically modified AD-MSC and BM-MSC using a retroviral system coding for full-length human PEDF gene aiming to use them as a platform to study PEDF's biological functions.

MATERIALS & METHODS

Cell Lines

The embryonic kidney fibroblast cells 293T, a highly human transfectable derivative of the 293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted, was maintained in culture in Dulbecco's Modified Eagle Medium (DMEM; Gibco) High Glucose supplemented with 10% heat inactivated FBS (Gibco), 1% L-glutamine (200 mM) (Lonza) and 1% penicillin-streptomycin (10^4 UI/ml and 10 mg/ml) (Carlo Erba). FLYRD-18, a packaging cell line derived from the HT1080 human osteosarcoma line, was maintained in culture in DMEM High Glucose (Gibco) supplemented with 10% heat inactivated FBS (Hyclone), 1% L-glutamine (200 mM) (Lonza) and 1% penicillin-streptomycin (10^4 UI/mL and 10 mg/mL) (Carlo Erba).

Isolation and Expansion of BM-MSC

After local anesthesia, BM tissue samples, of consenting donors was harvested from the iliac crest in accordance with local ethical committee guidelines. Standard procedure is described as follows: mononuclear cells (MNCs) were isolated from about 10 mL of BM tissue by the Ficoll density gradient method. Initially the sample was diluted 1:1 with 1X phosphate buffer saline (PBS) w/o Ca^{2+} and Mg^{2+} (Biochrom). The diluted sample was passed through a 19G needle for at least 20 times in order to obtain a single cell suspension. The diluted single cell suspension sample was gently transferred into tubes each containing Ficoll. After centrifugation at 700 x g for 20 minutes the MNCs were collected from the interface, washed in 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom), and re-collected after centrifugation at 300 x g for 10 minutes. The cells were then re-suspended in culture maintenance medium (MM) at a density of $7x10^3$ cells/cm^2 in culture flasks (Greiner Bio-one). MM consisted of alpha-Minimum Essential Media (α-MEM; Gibco) without nucleosides supplemented with 8% human platelet lysate (PLP), 1% L-Glutamine (200 mM) (Lonza), 0,2% heparin (Sigma) and 0,5% ciprofloxacin (2 mg/mL) (Fresenius Kabi).

Cells were incubated at 37°C with 5% CO_2 in a humidified incubator and were replenished with fresh medium 3 times per week until near confluent. When the adherent cells in the flask were confluent, cells were harvested, re-suspended, counted and re-plated.

Isolation and Expansion of AD-MSC

AD-MSC were obtained, as previously described [24], from lipoaspirate specimens of individuals undergoing liposuction for aesthetic purposes at the Plastic Surgery Unit University-Hospital of Modena and Reggio Emilia. Informed consent was obtained and study was approved from local Ethical Committee. After isolation, cells were seeded in maintenance media (MM), namely, alpha-Minimum Essential Media (α-MEM; Gibco) without nucleosides supplemented with 0,2% heparin (Sigma), 2,5% human platelet lysate (PLP), 1% L-Glutamine (200 mM) (Lonza) and 0,5% ciprofloxacin (2 mg/mL) (Fresenius Kabi). Media was replaced every 2-3 days discarding non adherent cells. Once confluent, the adherent AD-MSC were trypsinized with trypsin/EDTA (EuroClone), counted and seeded at $6x10^3$ cells/cm^2 in culture flasks (Greiner Bio-one). Cells were kept in incubators with controlled atmosphere (5% CO_2 and temperature of 37°C). This procedure was repeated for all the passages.

Retroviral Vector Production

Human PEDF cDNA sequence (accession number NM_002615) was cloned into an expression vector (13ADLASC_PEDF_nm24_pMA-RQ), this technique was performed by GeneArt (Life technologies). A bi-cistronic murine stem cell virus derived retroviral vector (pMIGR1) encoding the green fluorescence protein (GFP) was modified in our laboratory as follows: Above mentioned GeneArt expression vector and pMIGR1-GFP were digested separately using *EcoR1* restriction enzyme (Roche). On completion of the reaction the digested products were charged on

to a 1% agarose gel containing 0,5% ethidium bromide and specific DNA bands were visualized under an ultraviolet transilluminator. The digested products were subsequently purified using Gel purification kit (Qiagen) as per manufacturer's instructions. To prevent digested pMIGR1-GFP from re-circularizing it was dephosphorylated using *Shrimp Alkaline dephosphatase* (Roche). Following dephosphorylation, the purified full-length human PEDF cDNA fragment was cloned into the dephosphorylated pMIGR1-GFP vector using DNA ligation kit (Thermo Scientific) as per manufacturer's instructions. The resulting vector was defined as MIGR1-PEDF-GFP, while the empty MIGR1-GFP vector was used as control (Figure 3). To ensure that our MIGR1-PEDF-GFP vector was in the correct orientation and devoid of mutations it was sequenced (BigDye Terminator v.1 Cycle Sequencing Kit, Applied Biosystems) using specific primers (Table 1).

Creation of Retroviral Packaging Cell Line

Production of retroviral particles was performed as described: 293T cells were transiently transfected with JETPEI (PolyPLUS-transfection) with a mixture of helper plasmids (pSRα, pMD) and MIGR1-PEDF-GFP or MIGR1-GFP, as control. Retroviral particles from transiently transfected 293T cells were collected 48 hours after transfection and used to transduce the amphotropic packaging cell line FLYRD-18 with 6 μg/mL polybrene (Sigma). Three rounds of transduction were performed and the transduced FLYRD-18 cells thus obtained were defined as FLYRD-18-PEDF and FLYRD-18-GFP, respectively. Both packaging cell lines were expanded and then analyzed for GFP expression by FACS analyses using a FACSAria flow cytometer (Becton Dickinson) and viral titration was performed ($9x10^4$ TU/mL for FLYRD-18-PEDF and $5,9x10^5$ TU/mL for FLYRD-18-GFP cell lines).

Retroviral Transduction of AD-MSC and BM-MSC

Conditioned medium containing retrovirus from stably transduced FLYRD-18 expressing-PEDF cells was collected and used to infect AD-MSC and BM-MSC. AD-MSC were seeded at $4{,}2x10^3$ cells/cm^2 while BM-MSC were seeded at $5x10^3$ cells/cm^2 and were treated with an infectious medium containing 6 μg/mL polybrene (Sigma) and maintained for 6 hours in a humidified incubator at 37°C, 5% CO_2. Three rounds of transduction were performed. Cells were then kept in maintenance media (MM) i.e. for AD-MSC it was α-MEM (Gibco) without nucleosides supplemented with 1% L-Glutamine (Lonza), 0,5% Ciprofloxacin (Fresenius Kabi), 0,2% Heparin (Sigma) and 2,5% PLP whereas for BM-MSC it was α-MEM (Gibco) without nucleosides supplemented with 1% L-Glutamine (Lonza), 0,5% Ciprofloxacin (Fresenius Kabi), 0,2% Heparin (Sigma) and 8% PLP few days and enhanced GFP-expressing MSC were detected by FACS analyses. Non-transduced and transduced MSC were also monitored microscopically to detect any visible changes in morphology (Axiovert 200M Zeiss).

FACS Analyses

Cell surface antigen expression was investigated in wild-type and gene-modified AD-MSC and BM-MSC as follows, each cell type was performed separately. Briefly, cells were detached from plastic support with trypsin/EDTA (EuroClone), counted and aliquoted in FACS analyses polypropylene tubes (200,000 cells/tube) (VWR). MSC were subsequently incubated in blocking buffer (100 μL for each 200,000 cells/tube) containing DMEM High Glucose (Gibco), 10% heat inactivated FBS (Gibco), 0,1 Sodium Azide and human immunoglobulin G (both from Sigma) and incubated for 20 minutes on ice. After 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom) wash, the cells were re-suspended in 1X PBS with 0,5% Bovine Serum Albumin (BSA; Sigma) and stained with the following monoclonal antibodies: APC-anti-CD45, APC-anti-CD14, PE-anti-CD14 (all

from Becton Dickinson); APC-anti-CD34, PE-anti-CD140b, PE-anti-CD73, PE-anti-HLA-DR (all from BD Pharmigen); APC-anti-CD146 (MACS, Miltenyi Biotec); APC-anti-CD90, PE-anti-CD105 (both from eBioscience), and the appropriate isotype controls (BD Biosciences and BioLegend) followed by incubation on ice for 30 minutes (in dark). After 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom) wash, the cells were fixed using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) and read on FACSAria flow cytometer (Becton Dickinson).

RNA Extraction and cDNA Synthesis

Total cellular RNA was isolated using a single-step method with TRIzol® (Invitrogen) according to manufacturer's instructions http://tools.invitrogen.com/content/sfs/manuals/trizol_reagent.pdf. Briefly, 1 mL of TRIzol® reagent per cm^2 was added to 1-2 million cells and incubated at room temperature (RT) for 5 minutes to permit the complete dissociation of nucleoprotein complexes. The samples were harvested and transferred into new microcentrifuge tubes; chloroform was added according to the volume of TRIzol® as indicated by the manufacturer. After brief shaking, the samples were then incubated for 2-3 minutes at RT and centrifuged at 12000 x g for 15 minutes at +4°C. The upper transparent aqueous phase was transferred into a fresh tube and isopropyl alcohol was added to precipitate the RNA. The samples were incubated for 10 minutes at RT and centrifuged at 12000 x g for 10 minutes at +4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol. The samples were mixed and centrifuged at 7500 x g for 5 minutes at +4°C. The RNA pellet was then dried, dissolved in RNase-free water and incubated at 55-60°C for 10 minutes. The RNA was then quantified using a spectrophotometer (GeneQuant pro, Amersham Biosciences).

First-strand complementary cDNA was synthesized from 2 µg of total RNA using a RevertAid H minus first-strand cDNA synthesis kit (Fermentas) http://www.thermoscientificbio.com/reverse-transcription-rtpcr-rtqpcr/revertaid-h-minus-first-strand-cdna-synthesis-kit/. First 2 µg of RNA, 1 µL of Random hexamer primer and nuclease free water up to a final volume of 12 µL were taken into a new microcentrifuge tube, briefly centrifuged and incubated at 65°C for 5 minutes. After 5 minutes the microcentrifuge tube was centrifuged again, placed on ice and 4 µL of 5X Reaction buffer, 1 µL of RiboLock RNase Inhibitor (20 U/µl), 2 µL of 10 mM dNTP Mix and 1 µL of RevertAid M-MuLV Reverse Transcriptase (200 U/µL) were added. The next step involved mixing and incubating the sample for 60 minutes at 60°C, followed by an incubation of 5 minutes at 25°C. The reaction was terminated by heating at 70°C for 5 minutes. The single strand cDNA was quantified by spectrophotometer (Beckman Coulter DU® 730) and was used to perform a PCR. Consequently, 10 ng of single stranded cDNA was also employed in a real-time PCR.

PCR

cDNA of non-transduced and transduced samples were taken. For PCR of the PEDF gene a Taq PCR Master Mix (Roche) was employed. The following primers (400 nM) were used for the detection of human PEDF mRNA: 5'-ATGCAGGCCCTGGTGCTACT-3' (sense) and 5'-TTAGGGGCCCCTGGGGTCCA-3' (antisense) which yielded a 1257 bp long product; and for human β-actin mRNA: 5'-TCACCCTGAAGTACCCCATC-3' (sense) and 5'-TAGCACAGCCTGGATAGCAA-3' (antisense) which yielded a 226 bp long product. Conditions for amplification were as follows: Human PEDF program - 94°C for 10 minutes, followed by 25 cycles of 95°C for 1 minute, 58°C for 50 seconds, 72°C for 1 minute, 72°C for 10 minutes. Human β-actin program – 95°C for 10 minutes, followed by 25 cycles of 95°C for 1 minute, 53°C for 45 seconds, 72°C for 1 minute, 72°C for 10 minutes. The PCR product was then

electrophoresed on a 1% agarose gel which contained 0,5% ethidium bromide and specific DNA bands were examined under an ultraviolet transilluminator.

Quantitative Real-Time PCR for PEDF Detection

Quantitative real-time PCR was performed using the Applied Biosystems StepOne™ Real-Time PCR System using the TaqMan® Universal PCR Master Mix reagent http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042996.pdf. The levels of PEDF gene expression in non-transduced and transduced AD-MSC and BM-MSC was quantified using TaqMan reagents. Total reaction volume of 10 μL containing 10-50 ng of cDNA, TaqMan Master Mix (Applied Biosystems), and 10X of reverse and forward primers with probe were used The quantification of gene expression for target gene and reference gene was performed in separate tubes. Forward and reverse primers were designed using http://test.idtdna.com/site website, and to be specific for mRNA rather than genomic DNA, it was ensured that they spanned an intron sequence, thus avoiding the possibility of confusion from primer amplification of spliced mRNA. Expression of the target gene was normalized to that of the endogenous reference β-actin gene. Conditions for amplification were 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Reporter was FAM and quencher was NFQ. The $2^{-\Delta\Delta Ct}$ (cycle threshold) method, was used to calculate relative expression levels of the target gene (PEDF) defined by the primers as mentioned in Table 2.

Ki-67

The proliferative rate of non-transduced and transduced AD-MSC and BM-MSC was performed by quantitative real-time PCR using the Applied Biosystems StepOne™ Real-Time PCR System

and the Fast SYBR® Green Master Mix reagent http://tools.invitrogen.com/content/sfs/manuals/cms_046776.pdf. Total reaction volume of 10 µL containing 10-100 ng of cDNA, Fast SYBR Green Master Mix (Applied Biosystems), and 300 nM of reverse and forward primers were used. The quantification of gene expression for target gene and reference gene was performed in separate tubes. Expression of the target gene was normalized to that of the endogenous reference human β-actin gene. Conditions for amplification were 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds and 1 cycle of 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. The $2^{-\Delta\Delta Ct}$ (cycle threshold) method, was used to calculate relative expression levels of the target gene (Ki-67) defined by the primers summarized in Table 3.

ELISA

Transduced AD-MSC and BM-MSC were seeded at $6x10^3$ cells/cm^2 into a T25 culture flask (Greiner Bio-one) and were kept in MM until confluent. When 90% confluent, culture media was removed, after 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom) wash cells were replenished with 2,5 mL of conditioned media containing low serum concentration. Conditioned media comprised of DMEM Low Glucose (Gibco) supplemented with 2% heat inactivated FBS (Hyclone), 1% Glutamine (200 mM) (Lonza) and 1% penicillin-streptomycin (10^4 UI/mL and 10 mg/mL) (Carlo Erba). Conditioned media obtained from AD-MSC+GFP, AD-MSC+PEDF, BM-MSC+GFP and BM-MSC+PEDF were collected at different time points (24, 48 and 72 hours) and analyzed using Human PEDF ELISA kit (BioProducts MD) according to manufacturer's instructions. Briefly, supernatants were treated with 8 mol/L urea on ice for 30 minutes. The urea-treated samples were diluted 1:500-1:2000 in assay diluent, immediately added to the antibody-coated wells, and incubated at 37°C for 1 hour. After five washes, 100 µL diluted biotinylated mouse

anti-human PEDF monoclonal antibody was added to each well and the wells were incubated at 37°C for 1 hour. Then, 100 µL diluted streptavidin-peroxidase conjugate was added and incubated at 37°C for 30 minutes. Following which tetramethylbenzidine in a proprietary buffer with enhancer was added to the wells and incubated at RT for 30 minutes. Lastly, 100 µL stop solution was added and the absorbance was immediately measured at 450 nm using a microplate reader. For standardization, the PEDF concentration was normalized to the protein concentration in the samples.

Differentiation Assays: Ex Vivo Adipogenic Differentiation

After 5-7 days AD-MSC were 80-85% confluent, cells were washed with 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom), the cells were detached using trypsin/EDTA (EuroClone) for less than 5 minutes at 37°C. DMEM High Glucose (Gibco) with 10% heat inactivated FBS (Gibco), 1% penicillin/streptomycin (Carla Erba) was used to stop the trypsin reaction. The cells were centrifuged at 1400 rpm for 5 minutes at RT. For the adipogenic differentiation experiments, the cells were re-suspended in MM and were plated at a seeding density of $1x10^4$ cells/cm^2 in 6-well multiwell plate (Costar® Corning) for Oil Red O staining. To test the adipogenic differentiation potential of transduced and non-transduced AD-MSC, cells were maintained in DMEM Low Glucose (Gibco), 1% Glutamine (Lonza), 1% penicillin/streptomycin (Carlo Erba) along with 10% rabbit serum, 5% Horse Serum (both from EuroClone) supplemented with dexamethasone (1 µM), indomethacin (60 µM), recombinant human insulin (10 µM) and isobutyl methyl xantine (0,5 mM) (all from Sigma). All culture plates were incubated at 37°C with 5% CO_2 in a humidified incubator. Cells were induced for 10 days and detection of lipid droplets was performed by Oil Red O staining (Sigma) and observed microscopically (Axiovert 200M Zeiss).

After 10 days of adipogenic induction the cultures were stained with Oil Red O in order to visualize lipid droplets. Briefly, culture wells were washed with 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom). Cells were fixed with vapors of Formaldehyde (Sigma) for 10 minutes and then washed with water for 2 minutes. Staining was performed by adding the Oil Red O solution (10 mg/mL Oil Red O in 70% Ethanol and Acetone) (all from Sigma) into the wells for 3 minutes and excessive stain was removed by washing with water. Cells were then counterstained with filtered Harris Hematoxylin (Bio Optica) for 30 seconds. Air-dried and observed microscopically (Axiovert 200M Zeiss).

Differentiation Assays: Ex Vivo Chondrogenic Differentiation

After 5-7 days the AD-MSC were 80-85% confluent, cells were washed with 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom), the cells were detached using trypsin/EDTA (EuroClone) for less than 5 minutes at 37°C. DMEM High Glucose (Gibco) with 10% heat inactivated FBS (Gibco), 1% penicillin/streptomycin (Carlo Erba) was used to stop the trypsin reaction. The cells were centrifuged at 1400 rpm, for 5 minutes at RT. For the chondrogenic differentiation experiments, the cells were re-suspended in 1 mL of MM at a density of $2x10^5$ cells in each 15 mL Falcon tube for Alcian Blue staining. Cells were pelleted by centrifuging the Falcon tube at 1200 rpm for 10 minutes at RT. All culture tubes were incubated at 37°C with 5% CO_2 in a humidified incubator (lid not closed). To test the chondrogenic differentiation potential of transduced and non-transduced AD-MSC, cells were maintained in DMEM High Glucose (Gibco), 1% penicillin/streptomycin (Carlo Erba) along with BMP-6 (500 ng/mL) and TGF-β (10 ng/mL) (both from Peprotech), BD ITS™+Premix (BD Biosciences), dexamethasone (100 nM), ascorbic acid-2-phosphate (0,2 mM), proline (40 μg/mL) (all from Sigma) and sodium pyruvate (100 μg/mL) (Biochrom). Every time the media was changed, the cells were re-centrifuged at 1200

rpm for 10 minutes at RT to pellet the cells to the bottom of the 15 mL conical tube and kept in an incubator with a controlled atmosphere (37°C, 5% CO_2); the medium was changed every 2 days, leaving the pellet undisturbed inside the tube. Thin paraffin sections (6-5 μm) were taken and chondrogenic differentiation was detected by Alcian blue staining and observed microscopically (Axiovert 200M Zeiss).

Induction lasted 21 days at which time pellets were fixed for 1 hour in 10% Formaldehyde (Sigma) and then dehydrated by serial passages into Ethanol (Sigma) at increasing concentrations, from 70% to 100%. Samples were then inserted into paraffin blocks and cut in thin sections (6-5 μm) onto microscope slides for Alcian Blue staining. Slides were deparaffinated with the Histo-C cleaning agent (Celltech) and rehydrated through passages into a decreasing concentration alcoholic ladder (from 100% Ethanol to 70% Ethanol). Sample sections were circled on the slide using a PAP Pen and then incubated with a 0,5 mg/mL Hyaluronidase (Sigma) in buffer phosphate solution (8 g/L NaCl, 2 g/L NaH_2PO_4, 0,3 g/L Na_2HPO_4) with 10 mg/mL BSA (Sigma). Slides were washed in water for 5 minutes and then immersed in a 3% Acetic Acid solution for few seconds. Staining with 1% Alcian Blue solution (10 mg/mL Alcian Blue in 3% Acetic Acid, pH 2.5) (all from Sigma) lasted 30 minutes and after a washing step in water, samples were counterstained for 5 minutes with Nuclear Fast Red solution (Sigma) and then washed in water. The procedure was completed by fast rehydration through the alcoholic ladder as already indicated. Slides were then observed microscopically (Axiovert 200M Zeiss).

Differentiation Assays: Ex Vivo Osteogenic Differentiation

After 5-7 days the AD-MSC and BM-MSC were 80-85% confluent, cells were washed with 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom), the cells were detached using trypsin/EDTA (EuroClone) for less than 5 minutes at 37°C. DMEM High Glucose (Gibco) with 10% heat inactivated FBS

(Gibco), 1% penicillin/streptomycin (Carlo Erba) was used to stop the trypsin reaction. The cells were centrifuged at 1400 rpm, for 5 minutes at RT. For the osteogenic differentiation experiments, the cells were re-suspended in MM and were plated at a seeding density of $1x10^4$ cells/cm^2 in 6-well multiwell plate (Costar® Corning) for von Kossa staining and one of the wells was used for RNA extraction. All culture plates were incubated at 37°C with 5% CO_2 in a humidified incubator. When the cells reached 85-90% confluence (≈4 days), osteogenic differentiation was induced by using Osteogenic induction Medium (OM) consisting of MM supplemented with osteogenic factors, namely; 10 mM β-Glycerophosphate, 0,1 mM ascorbic acid-2-phosphate, 10 nM Dexamethasone (all from Sigma) and on the 7^{th} day of induction 100 ng/ml recombinant human BMP-2 (Peprotech) was added to the osteogenic differentiation media (v/v) or 8% PLP. On the 14^{th} day of induction, phosphate and carbonate anions were detected by von Kossa staining and observed microscopically (Axiovert 200M Zeiss).

After 2 weeks of osteogenic induction the cultures were stained with von Kossa in order to visualize phosphate and carbonate anions [210, 211]. The induced monolayers were washed in 1X PBS w/o Ca^{2+} and Mg^{2+} (Biochrom) at RT for 5 minutes and fixed in ice-cold methanol (100% v/v) (Sigma) for 4 minutes. The cells were rinsed twice in distilled water and incubated with 0,8 μm filtered 1% silver nitrate (Sigma) for 30 minutes under an ultraviolet lamp [212]. Stained samples were washed twice in distilled water and visualized by 10X magnification using an inverted microscope (Axiovert 200M Zeiss). The area of dark positive von Kossa staining was calculated as a percentage of the total area using Image J software (http://rsb.info.nih.gov/ij/).

Quantitative Real-Time PCR Analysis of Osteogenic Biomarker Expression

Quantitative real-time PCR was performed using the Applied Biosystems StepOne™ Real-Time PCR System and the Fast SYBR® Green Master Mix reagent

http://tools.invitrogen.com/content/sfs/manuals/cms_046776.pdf. Total reaction volume of 10 µL containing 10-100 ng of cDNA, Fast SYBR Green Master Mix (Applied Biosystems), and 300 nM of reverse and forward primers was used. The genes assessed were: Collagen type I alpha I chain gene (COL1A1), collagen type I alpha II chain gene (COL1A2), decorin (DCN), runt related transcription factor 2 (Runx2), osteocalcin (OCN) and osteopontin (SPP1). The quantification of gene expression for each target gene and reference gene was performed in separate tubes. Forward and reverse primers were designed using http://test.idtdna.com/site website, and to be specific for mRNA rather than genomic DNA, it was ensured that they spanned an intron sequence, thus avoiding the possibility of confusion from primer amplification of spliced mRNA. Expression of the target gene was normalized to that of the endogenous reference β-actin gene. Conditions for amplification were 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds and 1 cycle of 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. The $2^{-\Delta\Delta Ct}$ (cycle threshold) method was used to calculate relative expression levels of the target genes defined by the primers summarized in Table 3.

Statistical Analyses

Data has been expressed as mean (± standard deviation). A two-tailed P value of ≤ 0,05 from Student's t-test was considered statistically significant by Excel 2010 software (Microsoft, Inc., Redmond, Washington).

RESULTS

Packaging Cell Line FLYRD-18 Express PEDF Transcript After Transduction

Retroviral particles from transiently transfected 293T cells were used to infect the osteosarcoma stable producer cell line FLYRD-18 pseudotyped for RD114. Transduced FLYRD-18 cells were defined as FLYRD-18+PEDF and FLYRD-18+GFP, respectively. Infected cells when visualized under a fluorescence microscope revealed their GFP positivity. FACS analyses confirmed that more than 98% of total cell population was positive for GFP (Figure 4). To evaluate endogenous mRNA expression of PEDF in infected FLYRD-18 cells, PEDF mRNA levels were analyzed by PCR. PEDF mRNA was detected in FLYRD-18+PEDF transduced cells only, while no PEDF mRNA was seen in FLYRD-18+GFP infected cells (Figure 5). Target gene expression was normalized to human β-actin.

AD-MSC and BM-MSC can be Genetically Modified by Retroviral Vector Expressing PEDF

Conditioned medium containing retrovirus from stably transduced FLYRD-18 expressing-PEDF cells and/or FLYRD-18 expressing-GFP (control) was collected and used to transduce both human AD-MSC and BM-MSC. Both MSC types were successfully transduced by retroviral vector encoding for full-length human PEDF (MIGR1-PEDF-GFP) and with control vector (MIGR1-GFP). Successfully transduced AD-MSC were defined as AD-MSC+PEDF and AD-MSC+GFP (as control), respectively. By fluorescence microscopy, infected AD-MSC demonstrated a strong GFP signal as confirmed after FACS analyses: in both cases > 98% GFP positivity was obtained (Figure 6a). Similarly, BM-MSC were also efficiently transduced and were named as BM-MSC+PEDF and BM-MSC+GFP (as control). FACS studies revealed that transduced BM-MSC were > 92% GFP positive (Figure 6b).

Transduced MSC Express Significant Levels of PEDF Transcript Without Morphological Changes

To quantify the endogenous expression of PEDF mRNA in MSC, we examined PEDF mRNA levels in both non-transduced and transduced AD-MSC and BM-MSC by a semi-quantitative real-time PCR. PEDF mRNA levels were 70-fold higher in AD-MSC infected with PEDF when compared to AD-MSC+GFP as positive control (*$p = 4{,}6x10^{-8}$; Figure 7a) whereas PEDF mRNA levels were 140-fold higher in BM-MSC+PEDF when compared to BM-MSC+GFP serving as positive control (*$p = 3{,}9x10^{-7}$; Figure 7b). In both studies, target gene expression was normalized to human β-actin. After transduction, we then wanted to check whether PEDF expression was altering basic MSC features. By microscopy we observed that both non-transduced and transduced AD-MSC and BM-MSC demonstrated typical spindle-shaped morphology. This indicated that, despite the infection with a retroviral vectors encoding target (PEDF) or control (GFP) genes, the AD-MSC and BM-MSC are still able to maintain their basic morphological phenotype (Figure 8a and 8b). These data suggest that both AD-MSC and BM-MSC infected cells are permissive for mRNA transcript production.

Transduced MSC Secrete High Levels of PEDF Protein

As described earlier, PEDF is a secreted protein. Thus, it is necessary to quantify the amount of PEDF protein that is released by the gene-modified cells into culture media. To do this, conditioned media was collected from AD-MSC+GFP and AD-MSC+PEDF at several time points. It was revealed that PEDF protein was stably secreted into culture media. PEDF protein levels were under the detection limit in AD-MSC+GFP cells. On the other hand, there was a gradual increase in PEDF protein production noted from 24 hours (89,4 ng/mL) up to 72 hours (338,2 ng/mL) in AD-MSC+PEDF cells. Correspondingly, conditioned media was also collected

from BM-MSC+GFP and BM-MSC+PEDF and a progressive PEDF protein production from 24 hours (101,6 ng/mL) until 72 hours (370 ng/mL) in BM-MSC+PEDF cells was noted. PEDF protein was under detection limit in BM-MSC+GFP cells. This confirmed that adipose and bone marrow PEDF infected cells stably produced high levels of PEDF protein when compared to GFP infected cells (control) respectively.

MSC Isolated from Adipose Tissue and Bone Marrow Exhibit an Expected Immunophenotype After Gene Modification

In the attempt to assess whether *in vitro* expansion could modify main biological features of AD-MSC and BM-MSC that were transduced with retroviral vectors encoding PEDF and/or GFP, we analyzed cells for known surface antigens [15] together with a larger panel of monoclonal antibodies. As shown in Fig. 9a-b, the cells had an overlapping antigenic profile for wild-type and gene-modified AD and BM-MSC. Both cell groups were positive for the main mesenchymal markers such as CD90, CD105, CD73, CD140b and lacked expression of hematopoietic antigens including CD45, CD34, CD31 and CD14. The values obtained for AD-MSC wild-type and gene-modified were statistically significant ($*p < 4x10^{-2}$ $**p < 1,1x10^{-2}$) while for BM-MSC wild-type and gene-modified were not. As reported by Delorme et al. CD146 could be considered as a marker for BM-MSC [59] our wild-type and gene-modified BM-MSC showed high expression of CD146. However, in literature no information is available about AD-MSC expressing CD146, we observed very low levels of CD146 in wild-type and gene-modified AD-MSC. Both AD-MSC and BM-MSC groups were negative for MHC class I antigens. As there are no specific markers available for identifying MSC, these characteristics satisfy minimal requirements for designating the cells used in this study as MSC as previously defined. In the present study the cells used were without any further purification.

PEDF Expression can Alter the Ki-67 Levels in MSC

The Ki-67 antigen (pKi-67) is a nuclear protein essential for cell cycle progression and is identified in all phases of the cell cycle except the G0-phase [213]. This nuclear protein is used diagnostically as a proliferation marker in different cancers [214]. Thus, to examine the proliferative capacity of our gene-modified AD-MSC and BM-MSC we analyzed the expression levels of Ki-67 using a semi-quantitative real-time PCR. AD-MSC infected with PEDF was 0,12-fold higher as compared to AD-MSC infected with GFP serving as positive control (Figure 10a), although the obtained value was not statistically significant. In addition, BM-MSC+PEDF transduced cells was 1,3-fold lower as analyzed against BM-MSC+GFP cells serving as positive control (Figure 10b) the value obtained was statistically significant ($*p = 7{,}7x10^{-3}$). In both studies, target gene expression was normalized to human β-actin.

Differentiation Potential of Wild-Type and Gene-Modified AD-MSC to Adipogenic Lineage

Having defined AD-MSC phenotype, we then sought to assess human AD-MSC adipogenic differentiation potential. Wild-type and gene-modified AD-MSC were incubated with specific culture media to induce adipogenic differentiation in comparison with uninduced controls. After 10 days of adipogenic induction both wild-type and gene-modified AD-MSC were able to differentiate towards adipogenic lineage. We were able to observe round lipid droplets inside the cells and Oil red O staining further confirmed the adipose commitment (Figure 11). As expected no staining is observed in undifferentiated cells.

Differentiation Potential of Wild-Type and Gene-Modified AD-MSC to Chondrogenic Lineage

In addition, we also studied human AD-MSC chondrogenic differentiation potential. Wild-type and gene-modified AD-MSC were incubated with specific culture media to induce chondrogenic differentiation in comparison with uninduced controls. After 21 days of chondrogenic induction, pellets of both wild-type and gene-modified AD-MSC were inserted into paraffin blocks. Following this step, thin paraffin sections were made so as to prepare slides for histological staining. Alcian blue staining highlighted the sulphated proteoglycans typical of the cartilaginous matrix while Fast Red staining revealed the nuclei of resident chondrocytes derived from wild-type and gene-modified AD-MSC (Figure 12). As expected no staining is observed in undifferentiated cells.

Differentiation Potential of Wild-Type and Gene-Modified MSC to Osteogenic Lineage

Lastly, wild-type and gene-modified AD-MSC and BM-MSC when cultured *in vitro* under osteogenic conditions were also able to differentiate towards osteogenic lineage. Von Kossa staining points out the presence of mineralized nodular structures in all considered cell types after osteogenic differentiation in AD-MSC (Figure 13a) and BM-MSC (Figure 13b). As expected no staining is observed in undifferentiated cells in both cases studied. Using the Image J software we were able to quantify the signal obtained after von Kossa staining. We observed a gradual increase in positively stained areas in all of the induced samples when compared to control/non-induced samples. AD-MSC infected with PEDF that were induced to differentiate under controlled conditions into the osteogenic lineage demonstrated 10-fold increase when compared to AD-MSC non-transduced cells and a 5-fold increase when compared to AD-MSC transduced with GFP (Figure 14a). Furthermore, using the same conditions as used for AD-MSC osteogenic

differentiation it is demonstrated that even BM-MSC transduced with PEDF showed a 6-fold increase when compared to BM-MSC non-transduced cells and a 4-fold increase when compared to BM-MSC transduced with GFP (Figure 14b). The values obtained were statistically significant for AD-MSC group (*$p < 2x10^{-4}$ **$p < 1,7x10^{-9}$ ***$p < 1x10^{-10}$) as well as for BM-MSC group (*$p < 2,7x10^{-8}$ **$p < 3,4x10^{-2}$) respectively. Thus, this data indicates that the above mentioned cell manipulations do not affect the main AD-MSC and BM-MSC features.

Gene Modification Induces the Expression of Osteogenic Genes

A semi-quantitative real-time PCR was performed for several early and late osteogenic genes, namely, COL1A1, COL1A2, Decorin, Runx2, Osteocalcin and Osteopontin. These studies confirmed a remarkable upregulation of COL1A2, Decorin, Runx2 and Osteopontin genes implied in osteogenic lineage for all the differentiated cell types seen in AD-MSC (Figure 15a). The values obtained were statistically significant (*$p < 1x10^{-2}$ **$p < 5,1x10^{-4}$ ***$p < 3x10^{-2}$). We can confirm that osteogenesis had occurred as the levels of Runx2 were upregulated, though further studies are warranted. Correspondingly, real-time PCR studies conducted in BM-MSC suggest a hike in the expression levels for Decorin and Osteocalcin for all the differentiated cell types shown in BM-MSC (Figure 15b) but were not statistically significant. Values obtained for BM-MSC was statistically significant between BM-MSC empty induced and BM-MSC+PEDF induced for Osteopontin gene only (*$p = 2x10^{-2}$). In both studies, target gene expression was normalized to human β-actin. Thus, the above findings support the notion that gene modification does not have an impact on the main AD-MSC and/or BM-MSC differentiation pathways. Collectively all these data indicate that the above mentioned cell manipulations do not affect the main MSC features.

DISCUSSION

Gene therapy is a promising novel therapeutic strategy for treatment of several heritable and nonheritable human diseases, including infections, degenerative disorders and cancer [215, 216]. MSC possess several unique properties making them ideally suited for cellular therapies/ regenerative medicine and as vehicles for gene and drug delivery. These include: (1) relative ease of isolation; (2) the ability to differentiate into a wide variety of functional cell types of mesenchymal origin; (3) extensively expandable in culture before loss of differentiation potential; (4) hypoimmunogenic, they can induce immunosuppression upon transplantation; (5) they have pronounced anti-inflammatory properties; (6) following systemic administration they can home to damaged tissues, tumors, and metastasis [217]; (7) they are robust cells that can resist hypoxic stress and radiation therapy; (8) they can be readily genetically engineered ex vivo; (9) a cell-based therapy invoking reciprocal cellular interactions can respond more dynamically to tumor progression; and (10) in addition to influencing tumor cells, MSC may more broadly influence the tumor microenvironment helping to restore more normal vasculature and tissue homeostasis following tumor regression.

MSC can be readily transduced by the major clinically prevalent viral vector systems including those based upon adenovirus, the murine retroviruses, lentiviruses and adeno-associated viruses (AAV) [217]. With the help of these viral vector systems MSC can efficiently express a wide range of cytoplasmic, membrane-bound and secreted protein products. MSC can also be manipulated using physical (e.g., electroporation) and/or chemical agents (e.g., calcium phosphate or polycations) to enable gene transfer albeit with poorer efficiency than viral vectors [218, 219]. General ease of transduction coupled with the subsequent ability to select and expand ex vivo only the gene-modified cells to generate adequate numbers for clinical application make MSC one of the most promising stem cell populations for use in gene therapy studies and trials.

Genetically engineered MSC have been used for the improvement of neural injury, cardiovascular events, bone and cartilage regeneration as well as counteracting liver, pancreas, lung and kidney disorders (D'souza et al. submitted March 2015). Use of MSC for therapeutic-gene delivery was originally proposed by Matthews and Keating [220, 221]. Initial experiments on virus-mediated transgene expression in MSC showed efficient cell transduction with retroviral vectors expressing LacZ or IL-3 genes, and no changes in differentiation potential of MSC after gene transfer was seen. Thereafter, different groups were able to detect systemic levels of human growth hormone or IL-3, produced by either canine- or murine-transduced MSC, up to several months after infusion [222].

MSC transduced with BMP-2 and BMP-4 have shown to successfully repair a variety of musculoskeletal defects in animal models as BMPs are potent inducers of osteogenic differentiation [223–225]. These cells not only themselves underwent differentiation but also stimulated the neighboring cells to participate in the repair process. MSC transduced to overproduce IL-10, suppressed collagen-induced arthritis in a mouse model [226]. In an osteopenia mouse model, mice receiving transduced MSC that had stable expression of BMP had increased bone density [227]. In a rat model for spinal cord injury, rats treated with MSC stably overexpressing brain-derived neurotrophic factor (BDNF) had a better overall outcome than rats administered with MSC alone [228].

Similarly, the only cure for type 1 diabetic patients is pancreatic transplantation. However, shortage of pancreas donors calls for the development of alternative cell-replacement therapy. Transdifferentiation of human BM-MSC into insulin producing cells by overexpression of pancreatic duodenal factor 1 (PDX1) has been achieved *in vitro* [229, 230]. Only 50% of the cells

expressed insulin and secreted it in response to glucose in culture, whereas other islet hormones were expressed by all cells. Because these cells did not differentiate completely *in vitro*, as determined by microarray, transplantation under the renal capsule in streptozotocin-diabetic immunodeficient mice induced further differentiation and resulted in the reduction of hyperglycemia and stabilization of blood glucose levels during the 5-week follow-up [229]. None of the transplanted cells were observed to migrate to the pancreas, signifying the advantage of site-specific transplantation and avoidance of unwanted effects due to homing to undamaged organs following systemic infusion.

Stem cell therapy, especially using MSC, has been also considered as a promising method for treating myocardial infarction (MI) [231]. Enhanced expression (100-fold) of secreted frizzled-related protein 2 (SFRP2) by Akt-overexpressing MSC determined a pro-survival effect on myocardium by increasing nuclear β-catenin, which activated anti-apoptotic gene transcription in ischemic cardiomyocytes [232]. Genetic modification of MSC with hypoxia-regulated heme oxygenase- 1 [233], Bcl-2 [234] and Akt1 [235] resulted in enhanced cell survival upon transplantation into animal models by inhibiting apoptosis thus representing a potential opportunity.

In addition, MSC expressing glucagon-like peptide-1 transplanted into an Alzheimer's disease mouse model led to a decrease in A-beta deposition in the brain [236]. Lastly, in a rat model for bladder outlet obstruction, rats receiving transduced MSC with stable overexpression of hepatocyte growth factor (HGF) had decreased collagen accumulation in the bladder [237]. These studies indicate that modified MSC are a useful and feasible vehicle for protein expression/delivery to target various diseases and tissues. MSC have also been used for tumor

delivery of immunostimulatory cytokines and chemokines such as IL-2 [238, 239], IFN-β [46, 47, 240], INF-α [241], CX3CL1 [242], and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [243–247], suicide genes including thymidine kinase [248], cytosine deaminase [44], and carboxyesterase [249], growth factor antagonists (NK4) [250], and oncolytic viruses [251, 252], taking advantage of their tumor-homing capacities after systemic administration, or administered by intra-tumoral inoculation. The independent utility of many of these agents for cancer therapy was often limited by both their short half-life *in vivo* and their pronounced toxicity on normal non-malignant cells within the body. Using MSC to deliver these therapeutics may help minimize such problems, since MSC can selectively migrate to the tumor site and release their therapeutic effects locally, thus greatly increasing the agent's concentration within the tumor and significantly lowering its systemic toxicity. Suitable modification of MSC with tailored viral vectors can cause engrafted MSC to release the therapeutic agent steadily, allowing a single administration to result in long-lasting effects.

There have been several approaches based on the modification of progenitor cells, MSC included, by PEDF to combine the multiple potentials of this "Swiss-knife" protein with the advantages of the uses of MSC (Table 4). It is known that PEDF has a short half-life thereby restricting its application in clinic [170]. Based on these evidences, efforts have been made to develop effective and wide-applied PEDF delivery strategies by means of gene therapy. Gene therapy–mediated expression is a practical and potentially cost-effective method for chronic delivery of anti-angiogenic agents. Viral-mediated gene transfer of PEDF prevented retinal neovascularization in a wide range of experimental animal models [200]. Additionally, the therapeutic potential of PEDF using PEDF-expressing vectors against a number of tumor models, both *in vitro* and *in vivo* have demonstrated inhibition of growth and the suppression of metastasis in cancers, such as

pancreatic carcinoma, prostate, lung, breast, melanoma, neuroblastoma and ovarian cancer [253] (Table 5). These studies indicate that there are at least three ways of increasing the availability of PEDF: increasing its endogenous synthesis, supplying PEDF by gene transfer, and supplying PEDF protein or small peptides derived from it. However, the use of viral vectors includes high level of gene shuttling ability, but suffers from the risk of de novo cancer initiation via recombination within the patient cell genome. Plasmid vectors are safer to use but need to be improved upon to increase transfection efficiency. DNA-mediated PEDF gene transfer experiments showed short-term attenuation of blood vessel growth of up to 50%, but problems and patient risks are often encountered using current DNA-mediated gene-transfer strategies. These include (1) obtaining clinically effective viral titers, (2) toxicity and immunogenicity as a result of the expression of viral genes, (3) stable transgene expression in individuals requiring long-term treatment, and (4) insertional mutagenesis by random viral integration into the host genome. Nonetheless, clinical phase I/II studies has been conducted by GenVec in patients suffering from wet age-related macular degeneration (AMD) using adenoviral vector armed with PEDF. Various concentrations right up to $10^{9.5}$ pu have been tested and no toxicities have been reported. However, this drug is not yet FDA approved [254].

Studies conducted in animal models for eye disorders showed that injecting MSC transduced with adenoviral vectors expressing PEDF displayed a therapeutic effect [255, 256]. In the study conducted by Arnold and colleagues they used rat BM-MSC and human BM-MSC that were transduced with adenoviral vector carrying the sequences for GFP and human PEDF. Gene-modified BM-MSC were injected into the subretinal space of Wistar rats and Dystrophic RCS rats. In their experiments, the GFP reporter was stable for a period of 2 months without any adverse effects such as tumor formation. Furthermore, gene-modified MSC coding for PEDF had

a more pronounced rescue effect compared to non-transduced cells. However, their experiments revealed that non-transduced MSC already had the potential to rescue photoreceptor cells without gene modification. This could be attributed to the endogenous production of PEDF by non-transduced BM-MSC which was 78,2 pg/mL, though this was 1000-fold lower compared to RPE cells which produced 100 ng/mL of PEDF protein. Nonetheless, their findings raise the possibility that MSC have the potential to replace diseased retinal pigment epithelial (RPE) cells and delivery of therapeutic genes such as PEDF may serve to protect photoreceptor cells from degeneration [255]. On the other hand, Hou and group demonstrated that mouse BM-MSC could be transduced with an adenoviral vector coding for human PEDF. Reporter GFP expression in MSC was detected within 24 hours and was found to be 73% as determined by flow cytometer. *In vitro* studies showed that adenoviral PEDF (AdPEDF) persisted in BM-MSC for at least 8 days with a maximum production of 90 ng/100μL detected within 24 hours. GFP labeled BM-MSC were intravenously injected into laser-induced choroidal neovascularization (CNV) mouse model and were found to migrate to the site of CNV lesions and appeared to participate in vascular structure formation. Based on these findings they utilized AdPEDF-transduced BM-MSC to inhibit CNV growth and they noticed a significant reduction in CNV thickness and diameter *In vivo* AdPEDF production was constant throughout the experimental period and was more than 4-fold higher (27 ng/eye) than the quantity required to elicit anti-angiogenic effects and CNV inhibition (6 ng/eye) [257]. Moreover, co-culture of RPE cells with AdPEDF-transduced BM-MSC increased the proliferation and migration rate of RPE cells *in vitro* thus suggesting that AdPEDF-transduced BM-MSC were chemotactic for RPE cells. Their findings confirm that MSC play a role in CNV and serve as a powerful delivery system for anti-angiogenic therapeutic agents in CNV [256].

In a study where MI was induced in C57BL/6 mice, it had been observed that MSC could ameliorate MI injury, but that MSC derived from older donors had reduced efficacy. Very relevant for our study, wild-type old MSC secreted higher levels of PEDF than younger ones. Furthermore, infarcts treated with transduced MSC that overexpressed PEDF contained fewer endothelial cells, vascular smooth muscle cells and macrophages but had increased number of fibroblasts [258]. These authors used mouse BM-MSC that were transduced with adenoviral vector coding for human PEDF (AdPEDF) and short hairpin RNA (shRNA) targeting mouse PEDF to respectively overexpress PEDF in young BM-MSC and knockdown PEDF expression in older BM-MSC. Flow cytometer demonstrated that 75% of total cell population was GFP positive. AdPEDF-transduced young BM-MSC lingered for 8 days *in vitro* whereas old BM-MSC that were subjected to PEDF knock-down, PEDF expression was significantly blocked and this continued for at least 8 days *in vitro*. ELISA studies showed that wild-type old BM-MSC under normoxia and hypoxia conditions produced higher PEDF protein levels compared to wild-type young BM-MSC. Using a MI mouse model, wild-type BM-MSC (old and young), transduced young BM-MSC (AdNull and AdPEDF) and transduced old BM-MSC (Adshcontrol and AdshPEDF) were intravenously injected. The PEDF levels were significantly greater in older BM-MSC groups, moreover in transduced old BM-MSC (AdshPEDF) the levels of PEDF were decreased when compared to old BM-MSC (Adshcontrol) reflecting the inhibitory effect of shRNA targeting PEDF *in vivo*. They concluded that it was the increased expression of PEDF in aged MSC that impaired their therapeutic efficacy. Collectively, these data indicate the need of a far better characterization of MSC producing PEDF for precise therapeutic applications.

MSC are multipotent elements having high ex vivo expansion and retain the ability to self-renew. While MSC can be isolated from different tissues and are currently used for regenerative

medicine, bone marrow and adipose tissues represent rich and more defined sources of MSC [13]. In addition, MSC are able to differentiate into a variety of cell types hence making them an attractive therapeutic tool for cell transplantation and tissue engineering. Most importantly, MSC can be readily transduced by viral vector systems to efficiently express a wide range of cytoplasmic, membrane-bound and secreted protein products. Keeping this valuable information in mind in this study we used MSC derived from adipose tissue as well as from bone marrow to be transduced by a retroviral vector expressing PEDF.

Considering the relevant action of gene-modified cells to deliver PEDF in the different contexts, we began our study by creating a retroviral vector coding for the human PEDF cDNA. The final retroviral vector was termed as MIGR1-PEDF-GFP while MIGR1-GFP served as control. Thanks to the creation of a stable packaging cell line, namely FLYRD-18+PEDF we ensured that the produced vector particles were able to efficiently modify two different MSC types.

AD-MSC and BM-MSC were infected to obtain AD-MSC+PEDF, AD-MSC+GFP, BM-MSC+PEDF and BM-MSC+GFP, respectively. AD-MSC+PEDF and AD-MSC+GFP were found to be more than 98% positive for GFP whereas our BM-MSC+PEDF and BM-MSC+GFP were found to be more than 92% positive for GFP. Semi-quantitative real-time PCR confirmed that AD-MSC+PEDF produced about 70-fold increase in PEDF mRNA expression when compared to AD-MSC+GFP while BM-MSC+PEDF produced about 140-fold increase in PEDF mRNA expression when compared to BM-MSC+GFP. In a study conducted by Gao and colleagues they transduced human BM-MSC using lentiviral vector coding for human PEDF. Using flow cytometer they demonstrated that 96% of total cell population were GFP positive [259]. Two other studies in which mouse BM-MSC were transduced using an adenoviral vector coding for human PEDF demonstrated that 73-75% of total cell population was GFP positive as

determined by flow cytometer [256, 258]. Lastly, Zolochevska and group showed that human AD-MSC were transduced with a lentiviral vector coding for human PEDF. By means of semi-quantitative real-time PCR they showed that AD-MSC+PEDF produced about 50-fold increase in PEDF mRNA expression when compared to AD-MSC+GFP [260]. Taking these findings into account we see that, despite using a retroviral vector our transduction percentages obtained using BM-MSC is in line with the studies conducted by Gao and colleagues but was much higher when compared to the result obtained by Hou and group and Liang et al. Moreover, the fold increase seen in PEDF mRNA expression from our transduced AD-MSC was higher compared to Zolochevska et al. reported data.

PEDF is a secreted protein for this reason, to have an efficient action, gene-modified MSC should be able to release PEDF. After 48 hours of incubation with conditioned media, our wild-type BM-MSC were able to release 0,3 ng/mL (300 pg/mL) and AD-MSC were able to release 3 ng/mL of PEDF protein respectively (data not shown). This is in line with what was observed by the studies conducted by Chen et al. for BM-MSC [261]. However, our wild-type AD-MSC produced higher amounts of PEDF compared to studies conducted by Zolochevska and group [260]. In these reported studies, the exact amount of PEDF protein secreted into the conditioned media from wild-type BM-MSC and AD-MSC is not mentioned but it seems that PEDF secreted from wild-type BM-MSC was less than 10 ng/mL [261] while it was less than 0,01 ng/mL (10 pg/mL) for wild-type AD-MSC [260]. This indicates that wild-type BM-MSC and AD-MSC do indeed secrete very low levels of PEDF protein. In fact, a study conducted by Chiellini and group identified a number of proteins in the secretome of human AD-MSC and by means of a tandem mass spectrometry they found that PEDF is among one of the several proteins that are secreted during adipogenic and osteogenic differentiation [262]. Whether PEDF plays a direct role in the

commitment and differentiation of MSC into adipocytes or osteoblasts has not yet been investigated. Furthermore, Famulla and colleagues confirmed that PEDF is one of the most abundant proteins secreted by human adipocytes (130 ng/mL). PEDF protein expression significantly increased during adipogenesis which was paralleled by PEDF secretion [136]. On the contrary, it has been also reported that in adipose tissue PEDF levels are down-regulated during differentiation process to mature adipocytes [141]. However, a recent study demonstrates that the liver and not adipose tissue is the main source of circulating PEDF levels in humans [137]. These discoveries indicate that although PEDF is produced in high amounts by adipocytes future studies are necessary to investigate the production and regulation of circulating PEDF levels by other tissues.

After gene modification, we were able to dramatically increase PEDF levels in both MSC types. Gene-modified AD-MSC+PEDF were able to stably secrete PEDF protein with levels that gradually increased from 24 hours (89,4 ng/mL) up to 72 hours (338,2 ng/mL). Similarly, our gene-modified BM-MSC+PEDF also secreted PEDF protein and the levels progressively increased from 24 hours (101,6 ng/mL) until 72 hours (370 ng/mL). Chen et al. demonstrated that mouse BM-MSC transduced with an adenoviral vector coding for human PEDF were able to secrete 78 ng/mL of PEDF protein into the conditioned media collected after 48 hours of incubation [261]. While Zolochevska and group showed that human AD-MSC transduced with a lentiviral vector coding for human PEDF were able to secrete 82 pg/mL of PEDF protein into the conditioned media collected after 24 hours of incubation [260]. These studies clearly demonstrate that our retroviral system is able to produce far higher amounts of PEDF protein from our gene-modified AD-MSC+PEDF and BM-MSC+PEDF cells. Also, our retroviral system produces high amounts of PEDF protein which can be detected up to 72 hours in comparison to the other studies

mentioned here. Literature clearly states that PEDF protein has a very short half-life. Our data does not indicate the stability of the protein but instead it supports the fact that PEDF protein keeps getting accumulated which can be estimated up till 72 hours.

It has been reported that gene modification can be associated with changes in transduced cell properties [263], for this reason basic MSC properties where considered after transduction. Microscopical analyses showed that genetically modified AD-MSC and/or BM-MSC revealed no drastic changes in their morphology. Similarly, cell surface antigen expression further demonstrated that gene-modified AD-MSC and/or BM-MSC were positive for typical mesenchymal markers and were negative for hematopoietic markers. The antigen expression obtained was statistically significant for AD-MSC but not for BM-MSC. These findings collectively indicate that PEDF transduction was associated with the accordance of the minimal criteria as suggested [15].

Proliferation is a pivotal property in cells to be introduced within cell/gene therapy [264]. For this reason proliferation level of gene-modified AD-MSC and BM-MSC were considered. Curiously, the proliferation rate by Ki-67 expression of AD-MSC+PEDF was higher compared to AD-MSC+GFP although the values were not statistically significant. On contrary, Ki-67 levels were lower in BM-MSC+PEDF when compared to BM-MSC+GFP. In literature, studies in which gene-modified BM-MSC and/or AD-MSC with PEDF were used (Table 4) proliferation studies were not conducted. Since wild-type AD-MSC and AD-MSC+PEDF secrete PEDF protein it could be possible that Ki-67 levels were higher in AD-MSC+PEDF group. But the decrease in Ki-67 levels in BM-MSC+PEDF group is unclear thus suggesting that our findings warrant further investigations.

After addressing morphology, immunophenotype and proliferation, we wanted to consider the differentiation capacities of transduced wild-type and gene-modified AD-MSC towards adipogenic and chondrogenic lineages. We confirm that wild-type and gene-modified AD-MSC were able to differentiate into adipocytes: we could clearly detect the presence of lipid droplets in the cytoplasm of induced cells. We also confirm that wild-type and gene-modified AD-MSC when maintained in chondrogenic induction media for 21 days were able to differentiate into chondrocytes. A study conducted by Zolochevska et al. showed that wild-type and gene-modified AD-MSC were able to differentiate towards adipogenic lineage [260] and they demonstrated the same features of lipid droplet detection within cell cytoplasm as we observed in our study. However, chondrogenic differentiation was not performed in their study. Thus, our study indicates that PEDF does not affect the ability of AD-MSC to differentiate towards both adipogenic and chondrogenic lineages.

PEDF has a relevant role in osteogenesis. PEDF is expressed by osteoblasts lining the bone spicules in the ossification zone of metaphyseal bone, as well as by osteoblasts lining cortical periosteum. This report suggested that PEDF may play a regulatory role in the processes of chondrocyte and osteoblastic differentiation, endochondral ossification, and bone remodeling during development of long bones [156]. More, Lord et al. provided further evidence of PEDF's role in bone using deer antler model [157]. These findings suggested roles for PEDF in cell differentiation as well as in maintenance of high growth rates in mesenchymal stem layer. Some recent evidence suggests that PEDF promotes the differentiation of mesenchymal stem cells to the osteoblast lineage and regulates genes involved in osteogenesis and mineralization. Conversely, the lack of PEDF promotes adipogenesis and decreases osteoblastogenesis [265, 266]. Our data on gene-modified AD-MSC were able to demonstrate that adipogenesis is not

increased after PEDF transduction.

However, relevant attention has been given to the osteogenic potential. For this reason both BM- and AD- derived MSC were challenged after PEDF transduction. Thus, wild-type and gene-modified AD/BM-MSC were differentiated towards osteogenic lineage.

We confirm that wild-type and gene-modified AD-MSC and/or BM-MSC when maintained in osteogenic induction media for 14 days were able to differentiate into osteocytes. We could clearly detect the presence of mineralized deposits in the cytoplasm of induced cells. Using image J software we quantified the percentage of positively stained areas and confirmed that there is a gradual increase between the induced groups. The values obtained were statistically significant for AD-MSC group as well as for BM-MSC group respectively. By means of semi-quantitative real-time PCR we analyzed the expression of several osteogenic genes. In AD-MSC group, the levels of COL1A2, Decorin, Runx2 and Osteopontin were upregulated and the values were statistically significant. The upregulation of Runx2 indicates that osteogenic differentiation had occurred. Furthermore, in BM-MSC group, the expression levels for Decorin and Osteocalcin were increased, but were not statistically significant. Also, Osteopontin levels were dramatically upregulated in induced wild-type BM-MSC and the value was found to be statistically significant. However, the results obtained demand further studies.

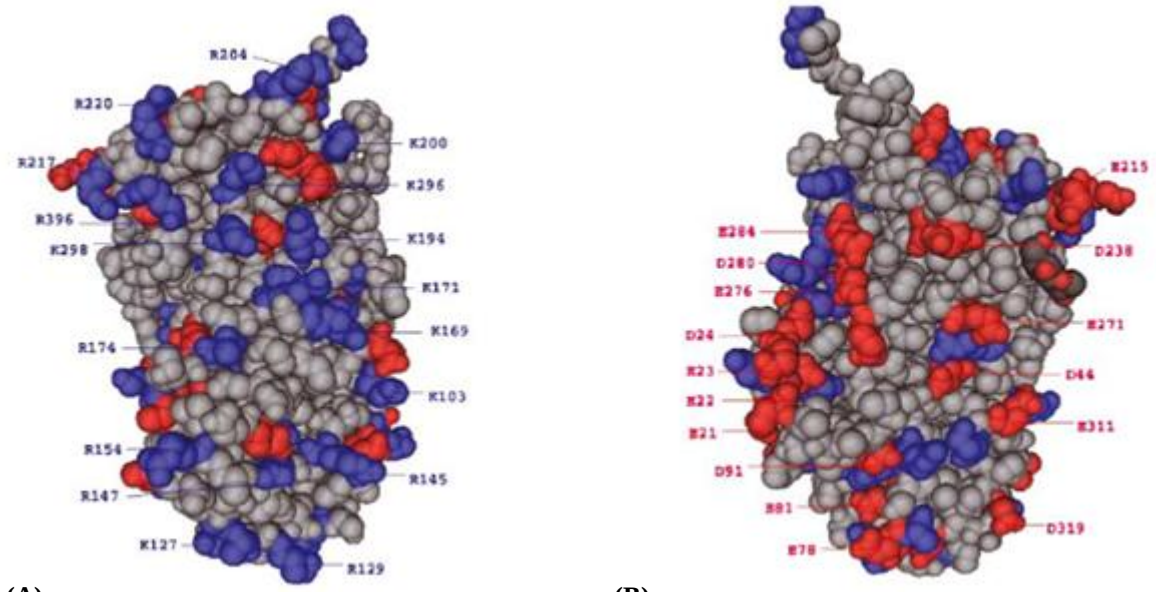
Li and colleagues showed that adding recombinant PEDF to the osteogenic induction media, the levels of osteogenic gene expression were regulated differently when analyzed at different time points [266]. In their study, human BM-MSC were incubated in osteogenic differentiating media in presence or absence of recombinant PEDF (250 ng/mL) for 3, 7, 14, 21 and 28 days. The osteogenic genes assessed were alkaline phosphatase (ALP), runt related transcription factor 2 (Runx2), osteopontin (OPN), osterix (OSX), osteocalcin (OCN), bone sialoprotein (BSP), bone morphogenetic protein-2 (BMP-2) and collagen type I alpha I chain gene (COL1A1). They

observed that ALP gene induction began at day 3 and its levels remained high until day 28. Runx2 levels were maximal at day 7 and then decreased with time. OSX levels were highest on days 14 and 21 and then decreased. OPN was expressed highly at day 3 but thereafter its levels decreased. OCN levels were highest at day 28 while BMP-2 was highest at day 7 and then its levels declined. Thus, the authors suggest that PEDF may accomplish MSC osteoblastic maturation by accelerating MSC differentiation through enhancing expression of genes that participate in osteoblastic differentiation. It should be noted that Li and colleagues used recombinant PEDF while our system involves AD-MSC and/or BM-MSC that have been genetically modified to overexpress PEDF.

Collectively, this PhD thesis was able to show that adult mesenchymal progenitor cells, namely MSC, can deliver PEDF thus representing a multipurpose therapeutic tool. Further studies and models can now be implemented on this technological platform based on MSC able to secrete relevant amounts of the protein to challenge the regenerative, anti-vascular and anti-tumoral potential of PEDF.

FIGURES & TABLES

Figure 1



(A) **(B)**

Figure 1: Crystal Structure of PEDF – (A) Space-filling model of human PEDF showing the cluster of positive charges on one face of the molecule. (B) Space-filling model of human PEDF showing the cluster of negative charges on the opposite face of the molecule. These charged groups are thought to interact with extracellular matrix molecules. Image is taken from [133]

Figure 2

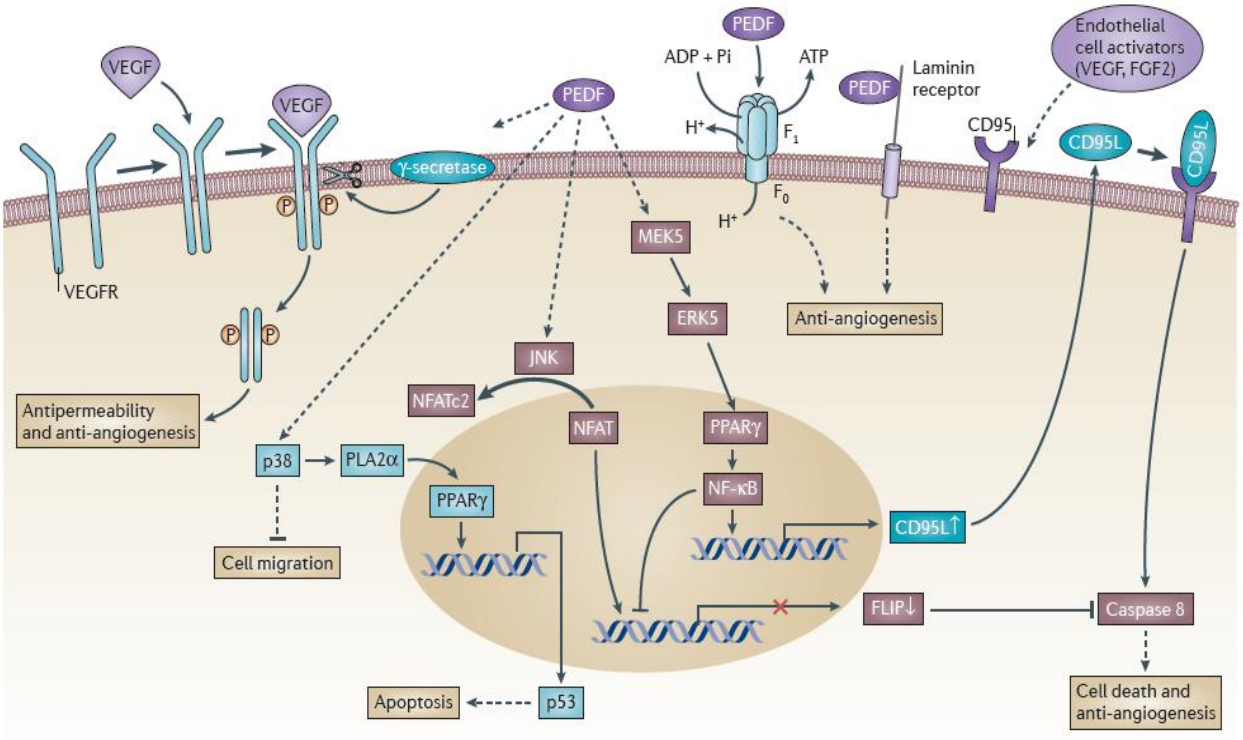


Figure 2: Signaling Events of PEDF in Endothelial Cells - During neovascularization, there is an activate interplay between PEDF and its receptors as well as between PEDF and several transcription factors that together regulate different functions such as anti-angiogenesis, inhibition of cell migration and induction of apoptosis in activated endothelial cells. Image is taken from [267]

Figure 3

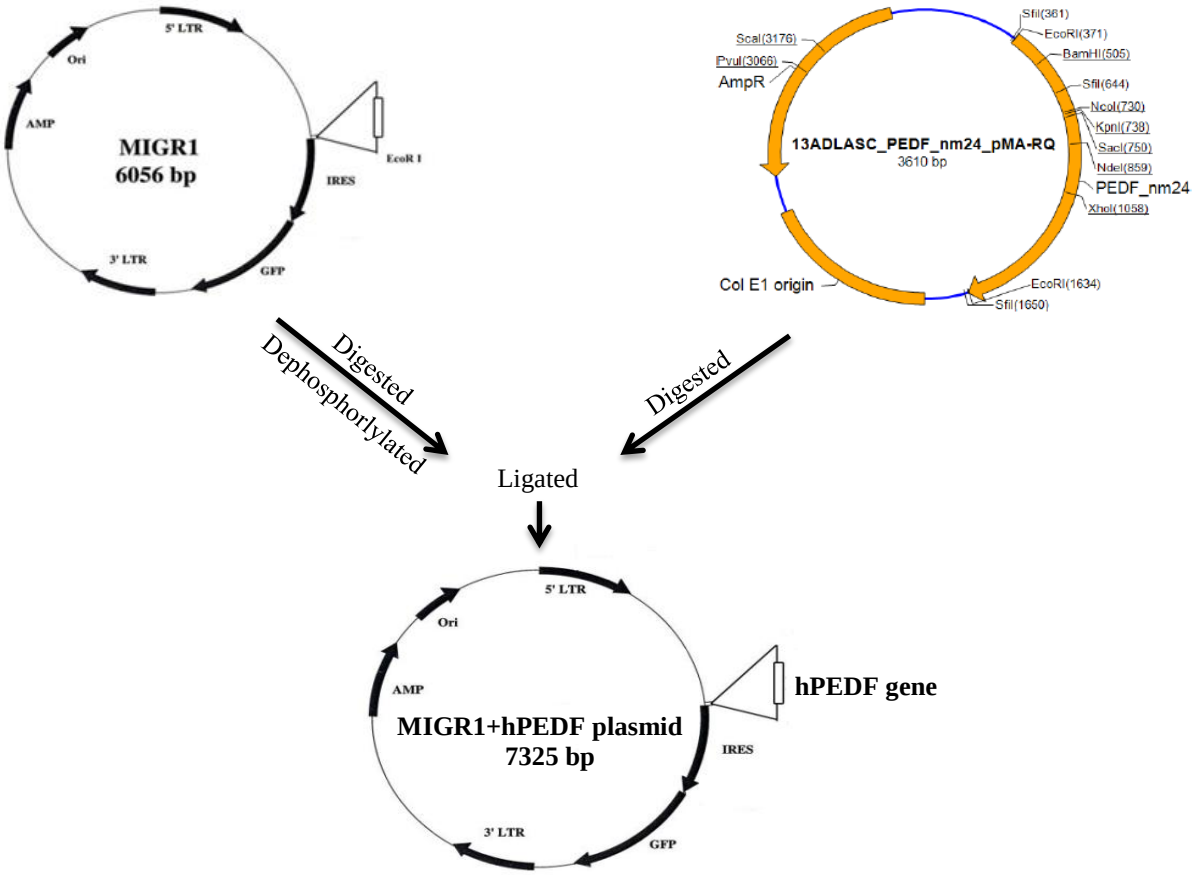


Figure 3: Creation of Retroviral Vector – Briefly, pMIGR1 and pMA-RQ+PEDF were digested using *EcoR1* restriction enzyme. Digested products were electrophoresed on to a 1% agarose gel and DNA bands were cut and purified. Purified pMIGR1 digest was dephosphorylated using *Shrimp Alkaline Dephosphatase* enzyme. Lastly, dephosphorylated pMIGR1 was then ligated with purified PEDF fragment to obtain pMIGR1+PEDF retroviral vector

Figure 4

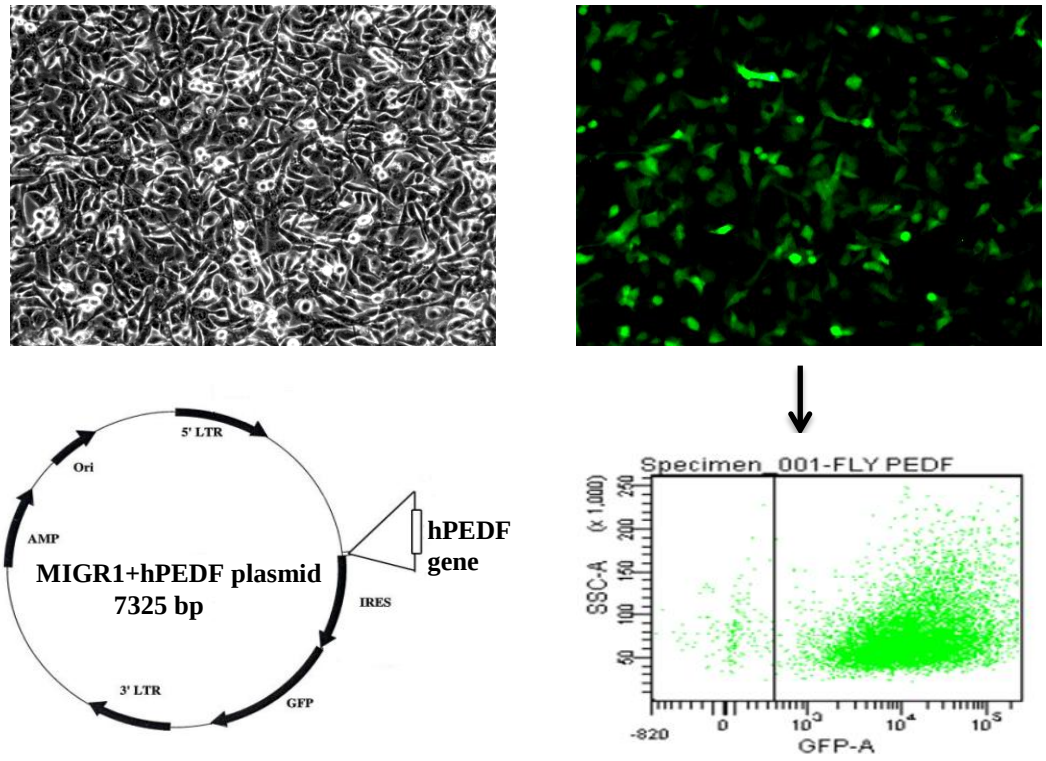


Figure 4: Transduction of FLYRD-18 Cells Using Retroviral Vector – FLYRD-18 cells were infected with pMIGR1+PEDF and the resultant cells were designated as FLYRD-18+PEDF and FLYRD-18+GFP served as control. Forty-eight hours after transduction, FLYRD-18+PEDF were monitored under a fluorescence microscope. Transduction efficiency of FLYRD-18+PEDF was determined using flow cytometry and was found to be > 98%

Figure 5

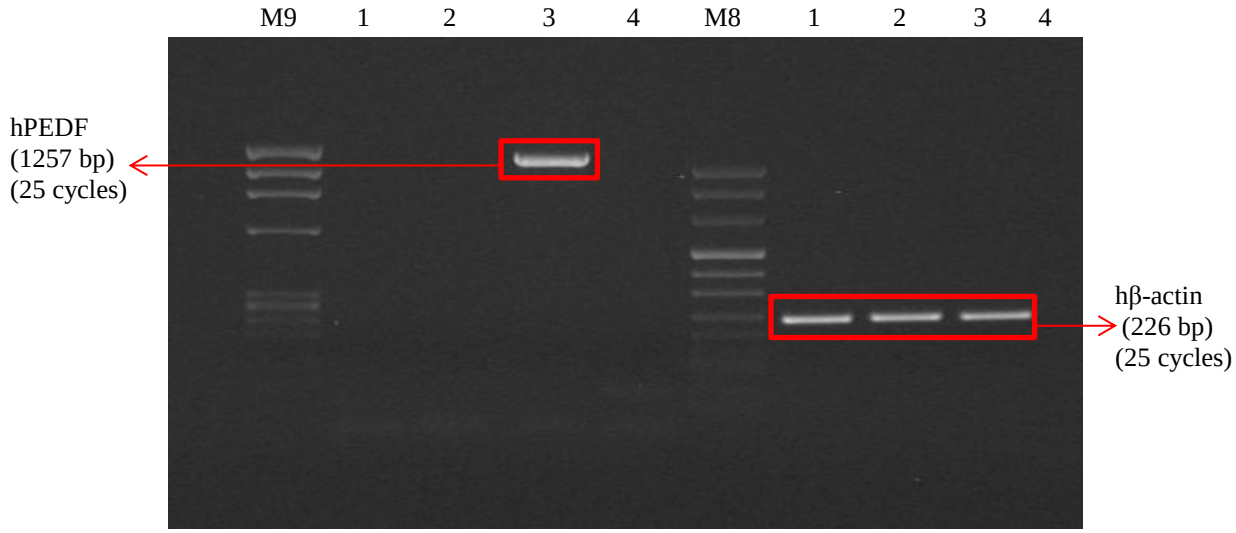


Figure 5: Presence of PEDF mRNA Transcript in FLYRD-18 Infected Cells - cDNA of transduced and non-transduced samples were taken and a PCR using human PEDF and human β-actin primers was performed. Left side of 1% agarose gel, strong human PEDF band corresponding to a length of 1257 bp was detected in FLYRD-18+PEDF lane only. No PEDF band seen in FLYRD-18 empty or FLYRD-18+GFP cells. Right side of 1% agarose gel, strong human β-actin band corresponding to a length of 226 bp was detected in all three cell groups tested. Key: M9 = Marker 9, M8 = Marker 8, 1 = FLYRD-18 empty, 2 = FLYRD-18+GFP, 3 = FLYRD-18+PEDF, 4 = Negative control

Figure 6

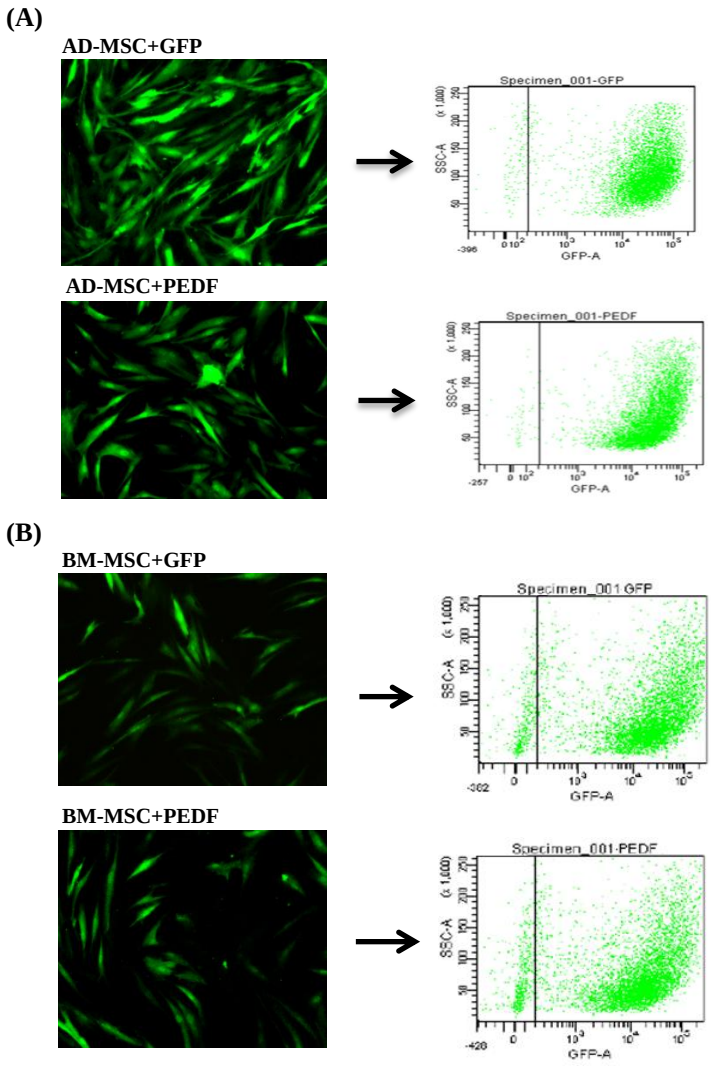


Figure 6: Transduction of MSC Using Retroviral Supernatant – (A) Human AD-MSC and (B) human BM-MSC were infected with pMIGR1+PEDF and pMIGR1+GFP. The resultant cells were designated as AD-MSC+GFP, AD-MSC+PEDF, BM-MSC+GFP, BM-MSC+PEDF. GFP infected cells served as control. By means of flow cytometer transduction efficiency of AD-MSC+GFP and AD-MSC+PEDF was found to be > 98% GFP positive, whereas for BM-MSC+GFP and BM-MSC+PEDF it was found > 92% GFP positive

Figure 7

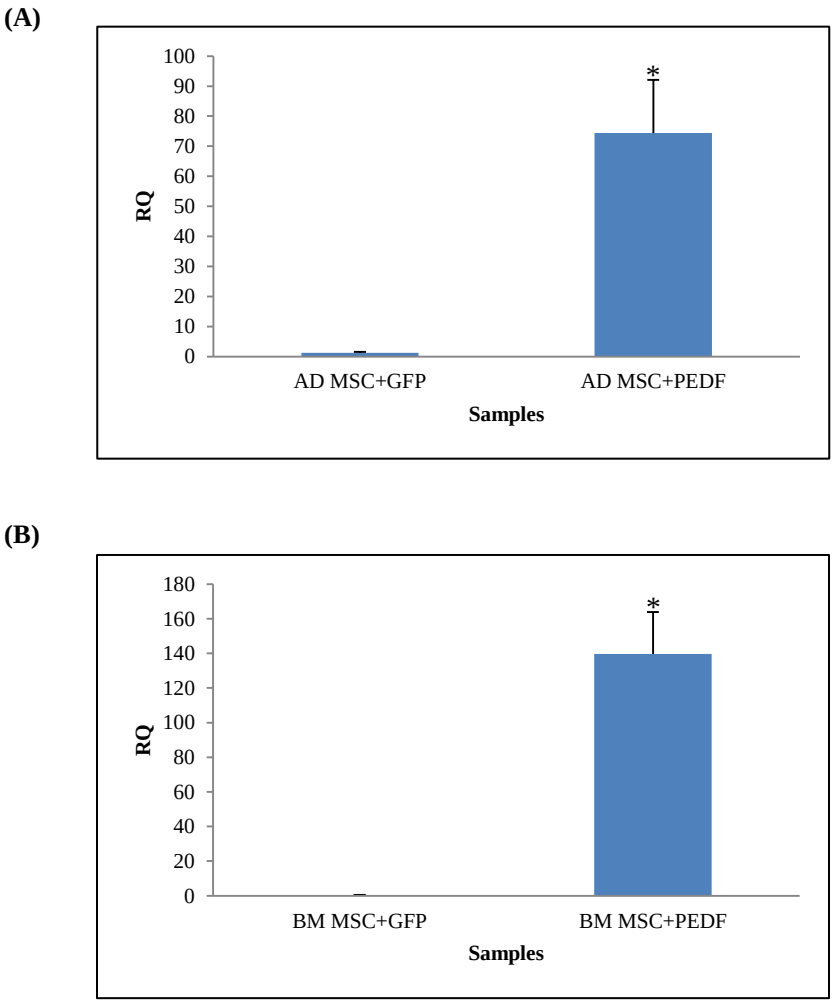


Figure 7: Quantitative Real-Time PCR Expression of PEDF in Infected MSC – Using cDNA (10-50 ng) a semi-quantitative real-time PCR was performed with PEDF specific primers in (A) AD-MSC and (B) BM-MSC that were transduced with a retroviral vector containing a marker gene (pMIGR1+GFP) or the same retroviral construct encoding PEDF cDNA. High levels of PEDF expression was detected in AD-MSC+PEDF and BM-MSC+PEDF infected cells when compared to AD-MSC+GFP and BM-MSC+GFP infected cells. The fold increase was also statistically significant for AD-MSC (*p = $4{,}6 \times 10^{-8}$) as well as for BM-MSC (*p = $3{,}9 \times 10^{-7}$) respectively

Figure 8

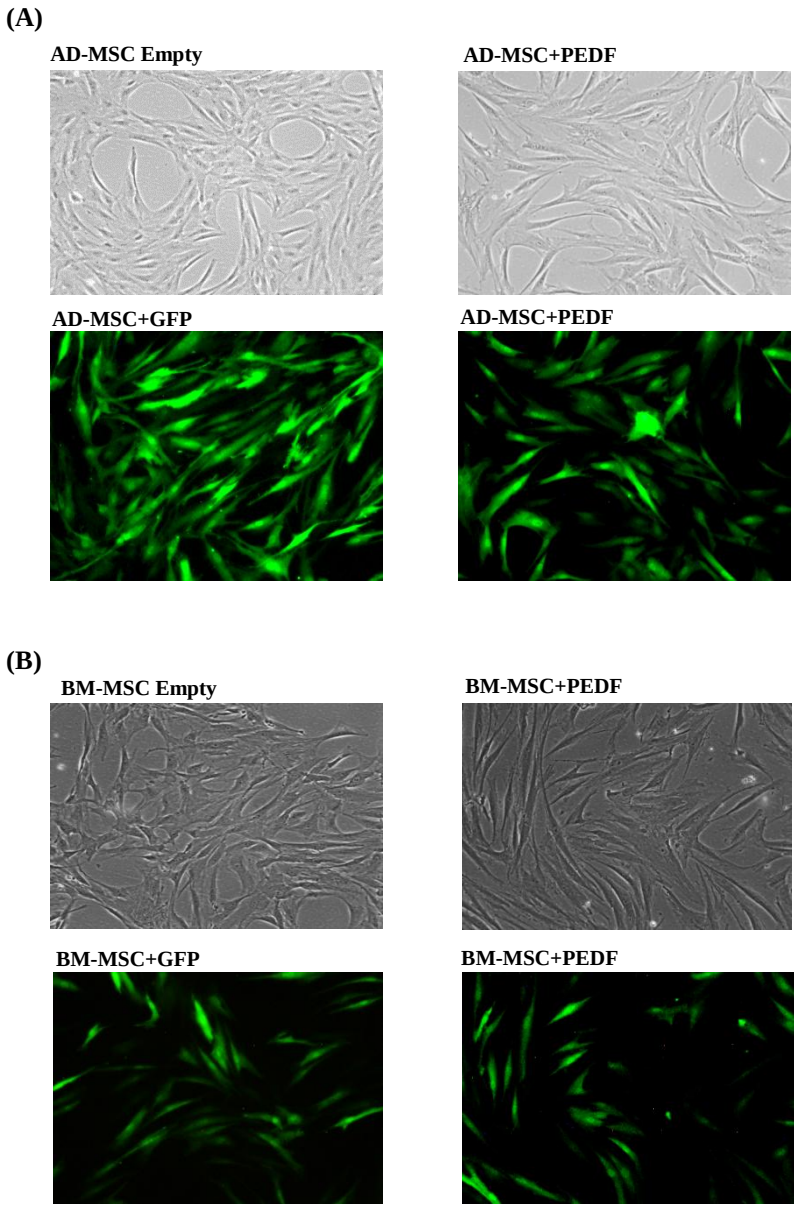
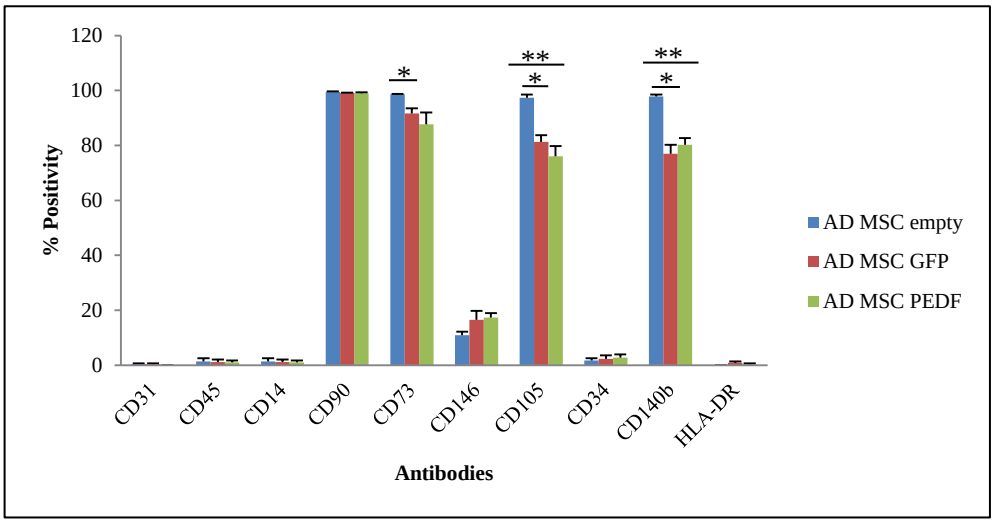


Figure 8: Morphological Analysis of MSC After Infection Using Retroviral Vector – (A) Human AD-MSC and (B) human BM-MSC were transduced with a retroviral vector containing a marker gene (pMIGR1+GFP) or the same retroviral construct encoding PEDF cDNA. Forty-eight hours after transduction, AD-MSC+GFP, AD-MSC+PEDF, BM-MSC+GFP and BM-MSC+PEDF were monitored under a fluorescence microscope. Wild-type and gene-modified AD-MSC as well as BM-MSC retained their spindle morphology and no drastic changes in their morphology were noted

Figure 9

(A)



(B)

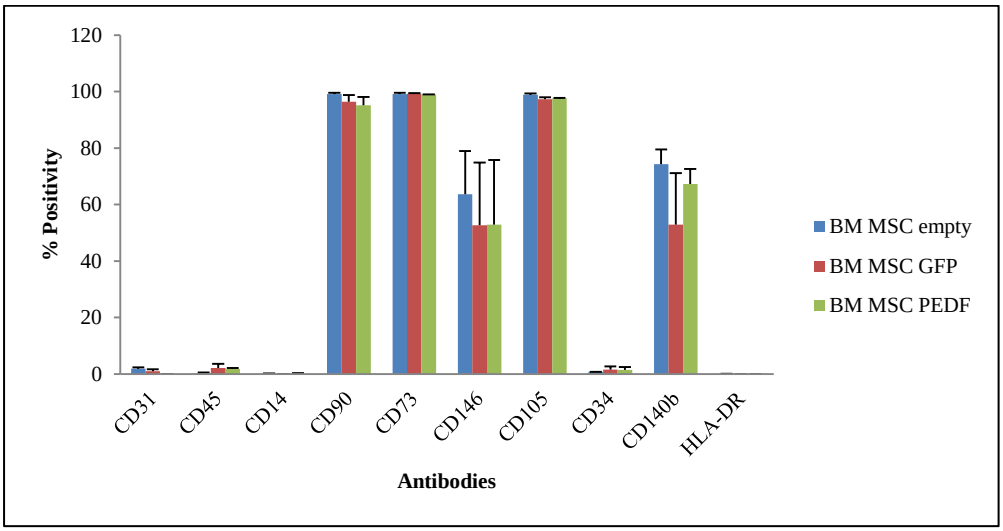
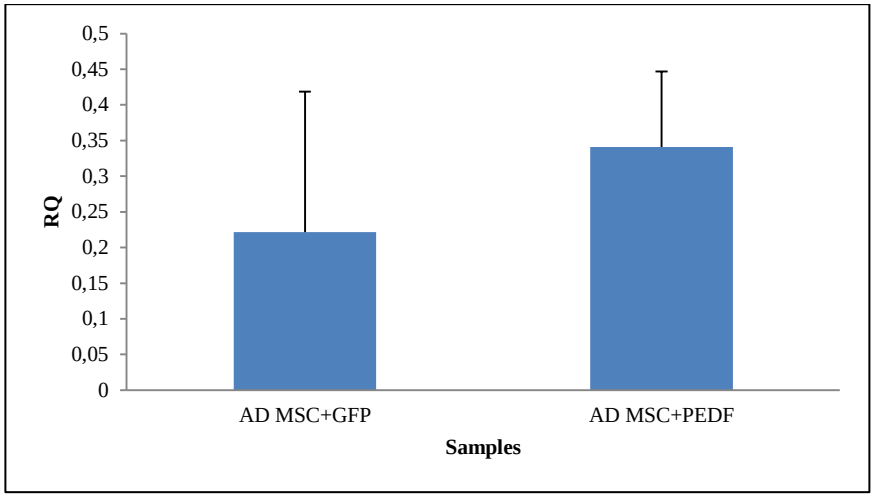


Figure 9: Fluorescence-Activated Cell Sorting Analysis of Wild-Type and Gene-Modified AD-MSC and BM-MSC - Cell surface antigen expression was demonstrated in (A) AD-MSC empty, AD-MSC+GFP and AD-MSC+PEDF and (B) BM-MSC empty, BM-MSC+GFP and BM-MSC+PEDF by well-defined hematopoietic (CD31, CD45, CD14, CD146 and CD34) and mesenchymal markers (CD73, CD90, CD105, CD140b) as per ISCT criteria using flow cytometry. The percent positivity for CD73, CD105 and CD140b were statistically significant (*$p < 4x10^{-2}$ **$p < 1,1x10^{-2}$) for AD-MSC samples but not for BM-MSC samples

Figure 10

(A)



(B)

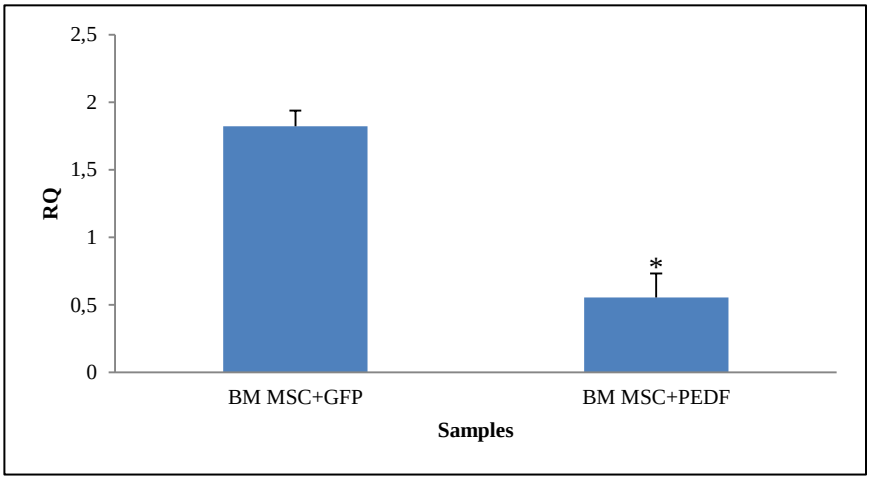


Figure 10: Quantitative Real-Time PCR Expression of Ki-67 in Infected MSC – Using cDNA (10-100 ng) a semi-quantitative real-time PCR was performed with Ki-67 specific primers in (A) AD-MSC and (B) BM-MSC infected with retroviral vectors coding for PEDF and/or GFP. Ki-67 expression levels were higher in AD-MSC+PEDF as compared to AD-MSC+GFP though value was not statistically significant. While in BM-MSC infected cells, Ki-67 expression levels were higher in BM-MSC+GFP compared to BM-MSC+PEDF and the value was statistically significant (*$p = 7{,}7 \times 10^{-3}$)

Figure 11

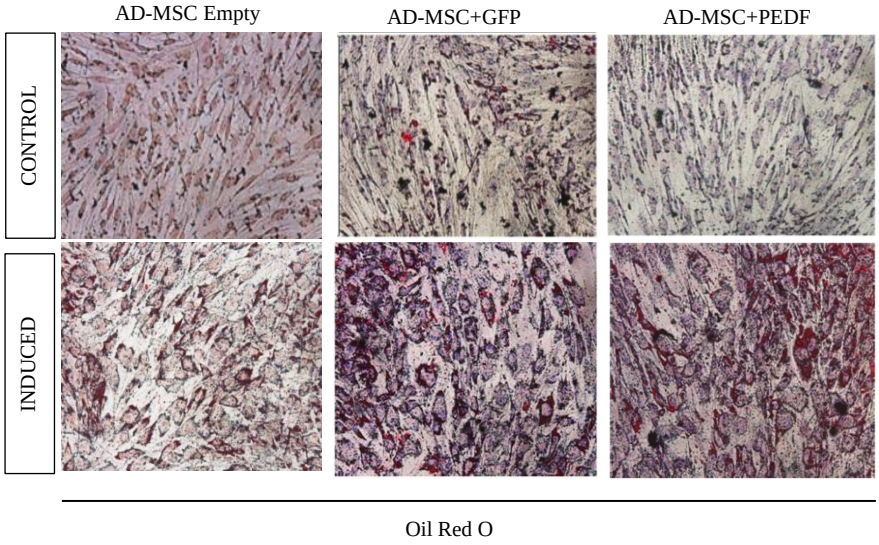


Figure 11: Adipogenic Differentiation of Wild-Type and Gene-Modified AD-MSC - Representative photomicrograph (10X) of stained samples has been shown in the upper and lower panel. Upper panel represents control conditions in which cells were maintained in normal culture media. After 10 days of induction wild-type and gene-modified AD-MSC were able to differentiate towards adipogenic lineage. In the lower panel lipid droplets were clearly seen in the cytoplasm of wild-type and gene-modified AD-MSC as detected by Oil Red O staining

Figure 12

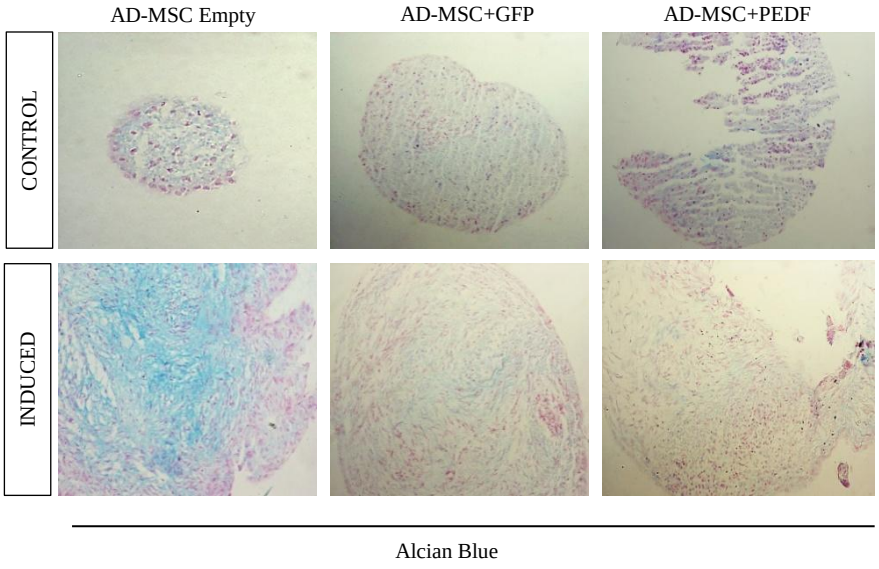
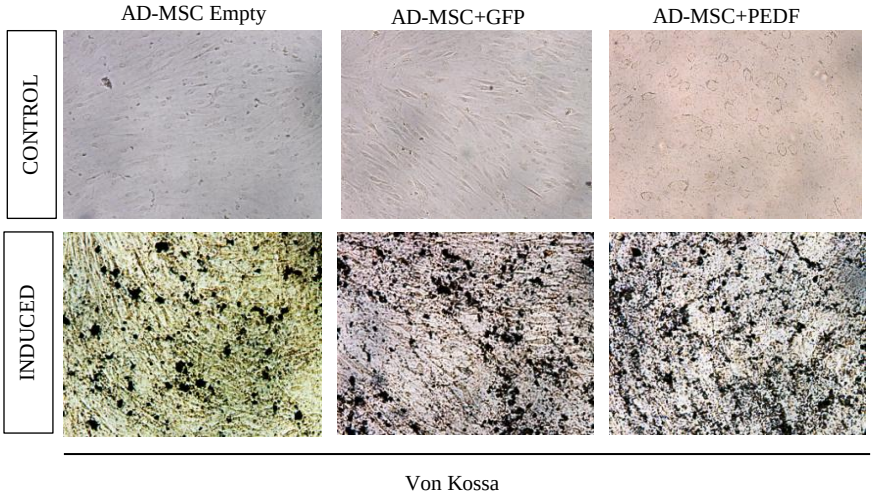


Figure 12: Chondrogenic Differentiation of Wild-Type and Gene-Modified AD-MSC - Representative photomicrograph (10X) of stained samples has been shown in the upper and lower panel. Upper panel represents control conditions in which cells were maintained in normal culture media. After 21 days of induction wild-type and gene-modified AD-MSC were able to differentiate towards chondrogenic lineage. In the lower panel chondrogenic differentiation was detected by Alcian Blue staining

Figure 13

(A)



(B)

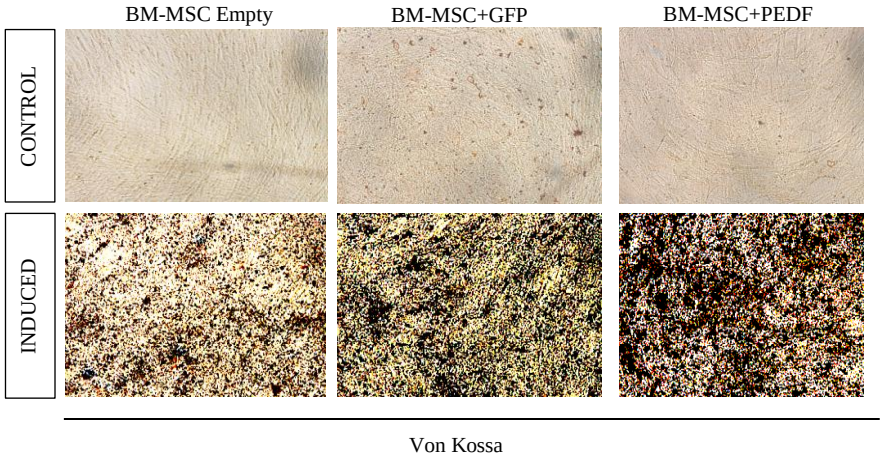
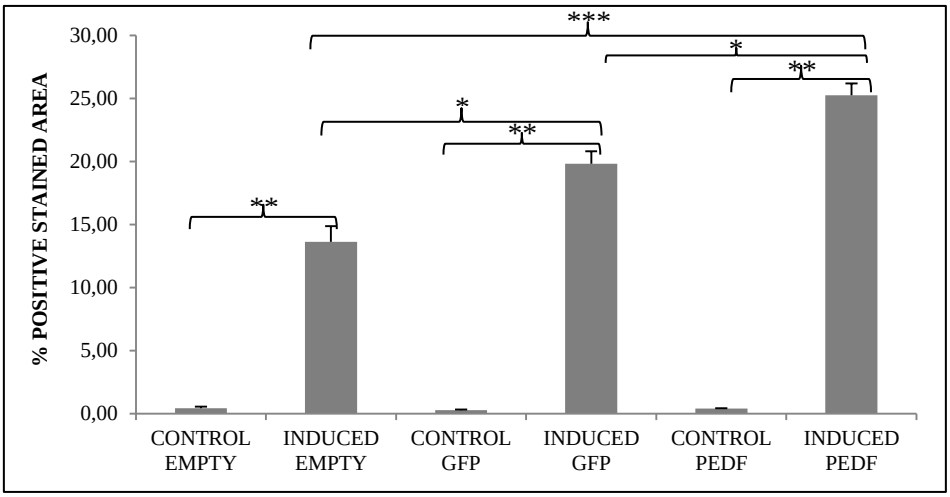


Figure 13: Osteogenic Differentiation of Wild-Type and Gene-Modified MSC – (A) AD-MSC and (B) BM-MSC. Representative photomicrograph (10X) of stained samples has been shown in the upper and lower panel. Upper panel represents control conditions in which cells were maintained in normal culture media. After 14 days of induction both AD-MSC as well as BM-MSC wild-type and gene-modified were able to differentiate towards osteogenic lineage. As shown in (A) and (B) the lower panel displays black particles of silver ions that were detected in both AD-MSC and BM-MSC wild-type and gene-modified using von Kossa staining

Figure 14

(A)



(B)

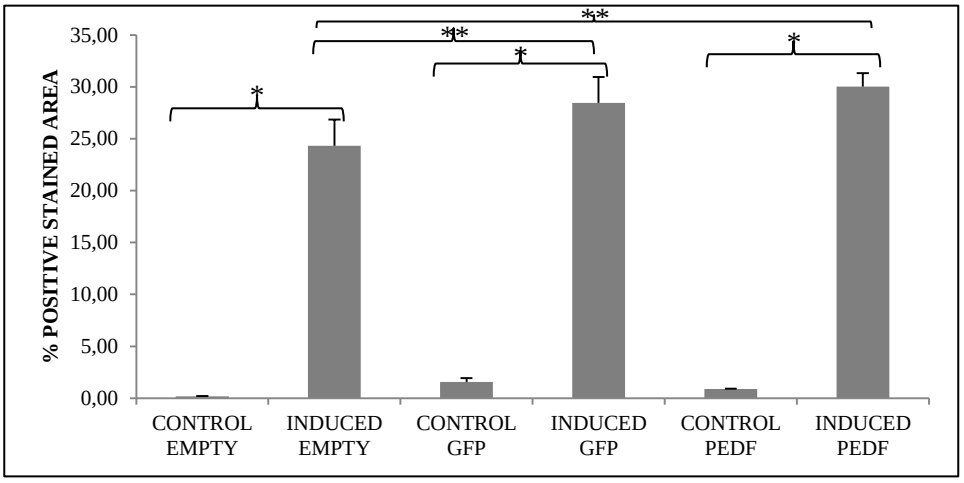
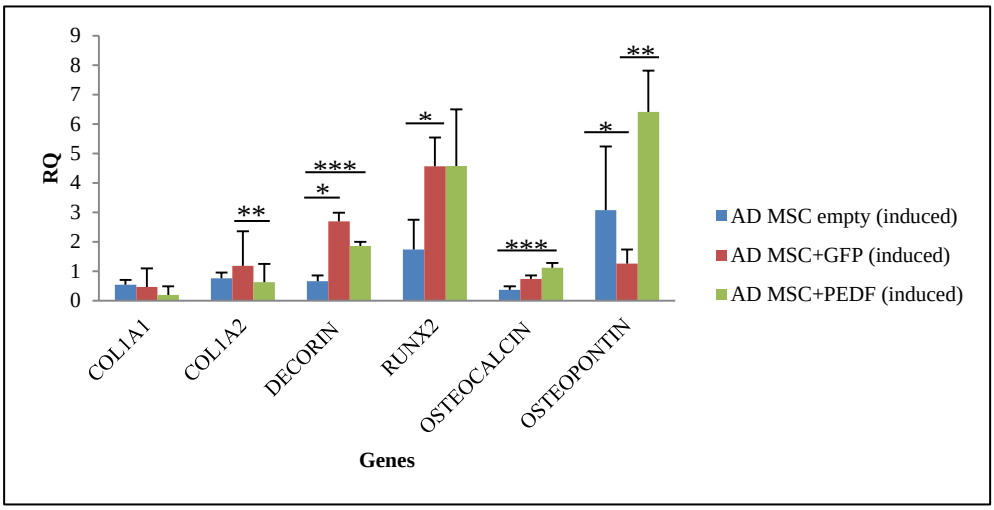


Figure 14: Quantification of von Kossa Staining in Wild-Type and Gene-Modified MSC After Osteogenic Differentiation – (A) AD-MSC and (B) BM-MSC. Using Image J software the von Kossa positive areas in photographs of osteogenic and control samples obtained after 2 weeks of osteogenic induction was evaluated. As shown in both graphs there is a gradual increase in the percentage of positively stained areas between induced empty, induced GFP and induced PEDF groups. The values obtained were statistically significant for AD-MSC group (*$p < 2x10^{-4}$ **$p < 1{,}7x10^{-9}$ ***$p < 1x10^{-10}$) as well as for BM-MSC group (*$p < 2{,}7x10^{-8}$ **$p < 3{,}4x10^{-2}$) respectively

Figure 15

(A)



(B)

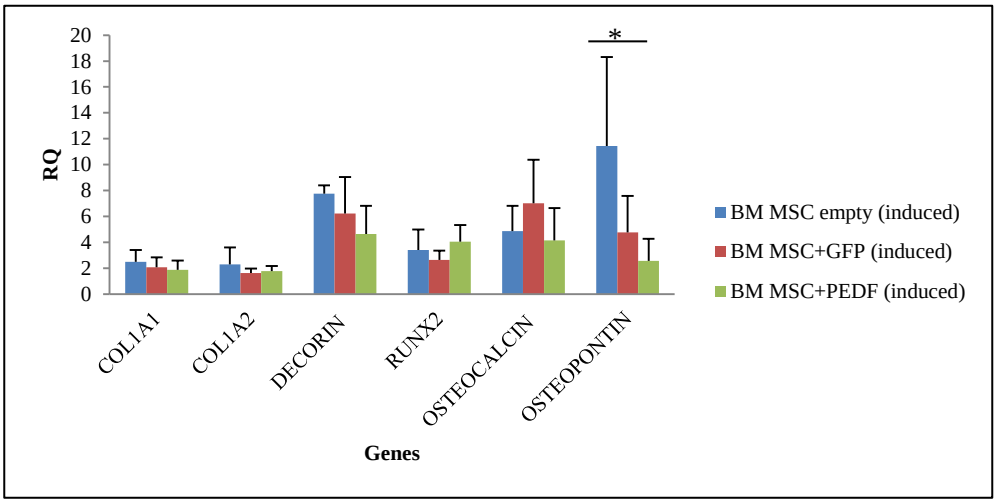


Figure 15: Expression of Osteogenic Genes in Wild-Type and Gene-Modified MSC After Osteogenic Differentiation – After 2 weeks of induction, molecular characterization of wild-type and gene-modified AD-MSC as well as for BM-MSC was conducted by means of semi-quantitative real-time PCR. (A) AD-MSC - Levels of COL1A2, Decorin, Runx2 and Osteopontin were upregulated and the values were statistically significant (*$p < 1x10^{-2}$ **$p < 5{,}1x10^{-4}$ ***$p < 3x10^{-2}$). (B) BM-MSC – Levels of Decorin, Osteocalcin and Osteopontin were upregulated but was statistically significant between BM-MSC empty induced and BM-MSC+PEDF induced for Osteopontin gene only (*$p = 2x10^{-2}$)

Table 1: Primer Sequences for the Retroviral Backbone Expressing PEDF

Region	Primer sequence
PEDF entire	5'- ATGCAGGCCC TGGTGCTACT-3' (sense) 5'- TTAGGGGCCCCTGGGGTCCA-3' (antisense)
PEDF internal	5'- CCGCTGGACTATCACCTTAA-3' (sense) 5'- CCGGTACAGGTCATAGCCGAA-3' (antisense)
MIGR1	5'- CCCTTGAACCTCCTCGTTCGAAC-3' (sense) 5'- AGAAGACAGGGCCAGGTTTCC-3' (antisense)

Table 2: Semi-Quantitative Real-Time PCR Primer Sequences for PEDF mRNA Expression

Gene		Primer sequence	Amplified length
PEDF	Probe	5'-56-FAM/CCTCACGGT/ZEN/CCTCTCTTCATCCAAGT/3IABkFQ/-3'	148 bp
	Primers	5'- GATCAGCATTCTCCTTCTCGG-3' 5'- TAAAACAGCCTTAGGGTCCG-3'	
β-actin	Probe	5'-56-FAM/TCATCCATG/ZEN/GTGAGCTGGCGG/3IABkFQ/-3'	110 bp
	Primers	5'- CCTTGCACATGCCGGAG-3' 5'- ACAGAGCCTCGCCTTTG-3'	

Table 3: Semi-Quantitative Real-Time PCR Primer Sequences

Gene	Primer sequence	Amplified length
β Actin	5'-ACCTTCTACAATGAGCTGCG-3' (sense) 5'-CCTGGATAGCAACGTACATGG-3' (antisense)	148 bp
COL1A1	5'-CCCCTGGAAAGAATGGAGATG-3' (sense) 5'-TCCAAACCACTGAAACCTCTG-3' (antisense)	148 bp
COL1A2	5'-AGGACAAGAAACACGTCTGG-3' (sense) 5'-GGTGATGTTCTGAGAGGCATAG-3' (antisense)	146 bp
DCN	5'-AAAATGCCCAAAACTCTTCAGG-3' (sense) 5'-GCCCCATTTTCAATTCCTGAG-3' (antisense)	146 bp
RUNX2	5'-TTCACCTTGACCATAACCGTC-3' (sense) 5'-GGCGGTCAGAGAACAAACTAG-3' (antisense)	148 bp
SPP1	5'-CAGTGATTTGCTTTTGCCTCC-3' (sense) 5'-ATTCTGCTTCTGAGATGGGTC-3' (antisense)	149 bp
OCN	5'-CAGCGAGGTAGTGAAGAGAC-3' (sense) 5'-TGAAAGCCGATGTGGTCAG-3' (antisense)	144bp
Ki-67	5'-GTCGTGTCTCAAGATCTAGCTTC-3' (sense) 5'-GTCATCTGCGGTACTGTCTTC-3' (antisense)	146 bp

Table 4: Therapeutic Potential of Gene-Modified MSC Transduced by Viral Vectors Expressing PEDF

MSC source	Viral vector	Tumors tested	Effect seen	Reference
BM (rat and human)	Adenoviral	None	Rescued photoreceptor cells	[255]
BM (mouse)	Adenoviral	None	Inhibits neovascularization	[256]
BM (mouse)	Adenoviral	None	Ameliorated myocardial infarction injury	[258]
BM (human)	Lentiviral	Hepatocellular carcinoma	Suppressed tumor growth and pulmonary metastases	[259]
AD (mouse and human)	Lentiviral	Prostate carcinoma	Tumor growth inhibited and prolonged survival	[260]
BM (human)	Adeno-associated	Glioma	Decreased angiogenesis and prolonged survival	[268]
BM (mouse)	Adenoviral	Lung carcinoma	Decreased tumor growth and angiogenesis, prolonged survival	[261]
Placenta (human)	Adenoviral	Melanoma	Decreased tumor growth and microvessel density, increased tumor apoptosis	[269]

Table 5: Therapeutic Potential of PEDF Against Several Cancers

Tumor tested	Effect seen	Reference
Lung cancer	Anti-angiogenic	[174, 270–275]
Bladder cancer	Anti-angiogenic	[276]
Breast cancer	Anti-angiogenic	[277–279]
Cervical cancer	Anti-angiogenic	[280, 281]
Colon adenocarcinoma	Direct tumor suppression, anti-angiogenic	[282, 283]
Colorectal	Anti-angiogenic	[283, 284]
Glioma	Anti-angiogenic	[285–287]
Hepatoblastoma	Anti-angiogenic	[288]
Hepatocellular carcinoma (HCC)	Anti-angiogenic	[140, 174]
Mesothelioma	Anti-angiogenic	[289]
Melanoma	Anti-angiogenic	[190, 290–292]
Neuroblastoma	Anti-angiogenic, neuronal cell differentiation	[158, 293]
Ovarian	Anti-angiogenic	[294]
Pancreatic	Direct tumor suppression, anti-angiogenic	[184, 295]
Prostate	Direct tumor suppression, anti-angiogenic, decreased cell viability, induced neuronal differentiation	[164, 184, 296–299]
Wilm's tumor	Anti-angiogenic	[183, 300]
Osteosarcoma	Direct tumor suppression, anti-angiogenic	[191–193, 301–306]

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BIBLIOGRAPHY

1. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV: **Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo**. *Transplantation* 1974, **17**:331–340.

2. Caplan AI: **Mesenchymal stem cells**. *J Orthop Res Off Publ Orthop Res Soc* 1991, **9**:641–650.

3. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: **Multilineage potential of adult human mesenchymal stem cells**. *Science* 1999, **284**:143–147.

4. Horwitz EM, Dominici M: **How do mesenchymal stromal cells exert their therapeutic benefit?**. *Cytotherapy* 2008, **10**:771–774.

5. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ: **Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice**. *Proc Natl Acad Sci U S A* 1995, **92**:4857–4861.

6. Gronthos S, Arthur A, Bartold PM, Shi S: **A method to isolate and culture expand human dental pulp stem cells**. *Methods Mol Biol Clifton NJ* 2011, **698**:107–121.

7. Zhang Q-Z, Su W-R, Shi S-H, Wilder-Smith P, Xiang AP, Wong A, Nguyen AL, Kwon CW, Le AD: **Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing**. *Stem Cells Dayt Ohio* 2010, **28**:1856–1868.

8. Kim J-S, Lee H-K, Kim M-R, Kim P-K, Kim C-W: **Differentially expressed proteins of mesenchymal stem cells derived from human cord blood (hUCB) during osteogenic differentiation**. *Biosci Biotechnol Biochem* 2008, **72**:2309–2317.

9. Rosada C, Justesen J, Melsvik D, Ebbesen P, Kassem M: **The human umbilical cord blood: a potential source for osteoblast progenitor cells**. *Calcif Tissue Int* 2003, **72**:135–142.

10. Fong CY, Richards M, Manasi N, Biswas A, Bongso A: **Comparative growth behaviour and characterization of stem cells from human Wharton's jelly**. *Reprod Biomed Online* 2007, **15**:708–718.

11. Fong CY, Gauthaman K, Cheyyatraivendran S, Lin HD, Biswas A, Bongso A: **Human umbilical cord Wharton's jelly stem cells and its conditioned medium support hematopoietic stem cell expansion ex vivo**. *J Cell Biochem* 2012, **113**:658–668.

12. Gucciardo L, Lories R, Ochsenbein-Kölble N, Done' E, Zwijsen A, Deprest J: **Fetal mesenchymal stem cells: isolation, properties and potential use in perinatology and regenerative medicine**. *BJOG Int J Obstet Gynaecol* 2009, **116**:166–172.

13. Bianco P, Robey PG, Saggio I, Riminucci M: **"Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease**. *Hum Gene Ther* 2010, **21**:1057–1066.

14. Zhang Y, Khan D, Delling J, Tobiasch E: **Mechanisms underlying the osteo- and adipo-differentiation of human mesenchymal stem cells**. *ScientificWorldJournal* 2012, **2012**:793823.

15. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: **Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement**. *Cytotherapy* 2006, **8**:315–317.

16. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC: **Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation**. *Transplantation* 2003, **75**:389–397.

17. Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J: **The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells**. *Biochem Biophys Res Commun* 2001, **289**:519–524.

18. Barry FP, Boynton RE, Haynesworth S, Murphy JM, Zaia J: **The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105)**. *Biochem Biophys Res Commun* 1999, **265**:134–139.

19. Haynesworth SE, Baber MA, Caplan AI: **Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies**. *Bone* 1992, **13**:69–80.

20. Simmons PJ, Torok-Storb B: **Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1**. *Blood* 1991, **78**:55–62.

21. Kemp KC, Hows J, Donaldson C: **Bone marrow-derived mesenchymal stem cells**. *Leuk Lymphoma* 2005, **46**:1531–1544.

22. Minguell JJ, Erices A, Conget P: **Mesenchymal stem cells**. *Exp Biol Med Maywood NJ* 2001, **226**:507–520.

23. Waller EK, Olweus J, Lund-Johansen F, Huang S, Nguyen M, Guo GR, Terstappen L: **The "common stem cell" hypothesis reevaluated: human fetal bone marrow contains separate populations of hematopoietic and stromal progenitors**. *Blood* 1995, **85**:2422–2435.

24. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH: **Human adipose tissue is a source of multipotent stem cells**. *Mol Biol Cell* 2002, **13**:4279–4295.

25. Olsen BR, Reginato AM, Wang W: **Bone development**. *Annu Rev Cell Dev Biol* 2000, **16**:191–220.

26. Liu L, Liu Z, Chen H, Zhang G, Kong Y, Kang X: **Oxidized low-density lipoprotein and β-glycerophosphate synergistically induce endothelial progenitor cell ossification**. *Acta Pharmacol Sin* 2011, **32**:1491–1497.

27. Choi K-M, Seo Y-K, Yoon H-H, Song K-Y, Kwon S-Y, Lee H-S, Park J-K: **Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation**. *J Biosci Bioeng* 2008, **105**:586–594.

28. Martins A, Duarte ARC, Faria S, Marques AP, Reis RL, Neves NM: **Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality**. *Biomaterials* 2010, **31**:5875–5885.

29. Jagodzinski M, Drescher M, Zeichen J, Hankemeier S, Krettek C, Bosch U, van Griensven M: **Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells**. *Eur Cell Mater* 2004, **7**:35–41; discussion 41.

30. Song I, Kim B-S, Kim C-S, Im G-I: **Effects of BMP-2 and vitamin D3 on the osteogenic differentiation of adipose stem cells**. *Biochem Biophys Res Commun* 2011, **408**:126–131.

31. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM: **Pluripotency of mesenchymal stem cells derived from adult marrow**. *Nature* 2002, **418**:41–49.

32. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP: **Bone marrow as a potential source of hepatic oval cells**. *Science* 1999, **284**:1168–1170.

33. Spees JL, Olson SD, Ylostalo J, Lynch PJ, Smith J, Perry A, Peister A, Wang MY, Prockop DJ: **Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma**. *Proc Natl Acad Sci U S A* 2003, **100**:2397–2402.

34. Woodbury D, Schwarz EJ, Prockop DJ, Black IB: **Adult rat and human bone marrow stromal cells differentiate into neurons**. *J Neurosci Res* 2000, **61**:364–370.

35. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A: **Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells**. *Br J Haematol* 2005, **129**:118–129.

36. Planat-Bénard V, Menard C, André M, Puceat M, Perez A, Garcia-Verdugo J-M, Pénicaud L, Casteilla L: **Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells**. *Circ Res* 2004, **94**:223–229.

37. Dennis JE, Merriam A, Awadallah A, Yoo JU, Johnstone B, Caplan AI: **A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse**. *J Bone Miner Res Off J Am Soc Bone Miner Res* 1999, **14**:700–709.

38. Phinney DG, Prockop DJ: **Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views**. *Stem Cells Dayt Ohio* 2007, **25**:2896–2902.

39. Nauta AJ, Fibbe WE: **Immunomodulatory properties of mesenchymal stromal cells**. *Blood* 2007, **110**:3499–3506.

40. Lange C, Tögel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, Westenfelder C: **Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion–induced acute renal failure in rats**. *Kidney Int* 2005, **68**:1613–1617.

41. Phinney DG, Isakova I: **Plasticity and therapeutic potential of mesenchymal stem cells in the nervous system**. *Curr Pharm Des* 2005, **11**:1255–1265.

42. Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, Brigham KL: **Bone marrow-derived mesenchymal stem cells in repair of the injured lung**. *Am J Respir Cell Mol Biol* 2005, **33**:145–152.

43. Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, Sato T, Miyanishi K, Takayama T, Takahashi M, Takimoto R, Iyama S, Matsunaga T, Ohtani S, Matsuura A, Hamada H, Niitsu Y: **Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion**. *Blood* 2005, **106**:756–763.

44. Kucerova L, Altanerova V, Matuskova M, Tyciakova S, Altaner C: **Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy**. *Cancer Res* 2007, **67**:6304–6313.

45. Shah K: **Mesenchymal stem cells engineered for cancer therapy**. *Adv Drug Deliv Rev* 2012, **64**:739–748.

46. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M: **Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors**. *Cancer Res* 2002, **62**:3603–3608.

47. Studeny M, Marini FC, Dembinski JL, Zompetta C, Cabreira-Hansen M, Bekele BN, Champlin RE, Andreeff M: **Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents**. *J Natl Cancer Inst* 2004, **96**:1593–1603.

48. Frenette PS, Pinho S, Lucas D, Scheiermann C: **Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine**. *Annu Rev Immunol* 2013, **31**:285–316.

49. Prockop DJ: **Marrow stromal cells as stem cells for nonhematopoietic tissues**. *Science* 1997, **276**:71–74.

50. Chen Q, Liu K, Robinson AR, Clauson CL, Blair HC, Robbins PD, Niedernhofer LJ, Ouyang H: **DNA damage drives accelerated bone aging via an NF-κB-dependent mechanism**. *J Bone Miner Res Off J Am Soc Bone Miner Res* 2013, **28**:1214–1228.

51. Karsenty G, Ferron M: **The contribution of bone to whole-organism physiology**. *Nature* 2012, **481**:314–320.

52. Schallmoser K, Rohde E, Reinisch A, Bartmann C, Thaler D, Drexler C, Obenauf AC, Lanzer G, Linkesch W, Strunk D: **Rapid large-scale expansion of functional mesenchymal stem cells from unmanipulated bone marrow without animal serum**. *Tissue Eng Part C Methods* 2008, **14**:185–196.

53. Pountos I, Corscadden D, Emery P, Giannoudis PV: **Mesenchymal stem cell tissue engineering: techniques for isolation, expansion and application**. *Injury* 2007, **38 Suppl 4**:S23–33.

54. Calloni R, Cordero EAA, Henriques JAP, Bonatto D: **Reviewing and updating the major molecular markers for stem cells**. *Stem Cells Dev* 2013, **22**:1455–1476.

55. Lv F-J, Tuan RS, Cheung KMC, Leung VYL: **Concise review: the surface markers and identity of human mesenchymal stem cells**. *Stem Cells Dayt Ohio* 2014, **32**:1408–1419.

56. Bara JJ, Richards RG, Alini M, Stoddart MJ: **Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic**. *Stem Cells Dayt Ohio* 2014, **32**:1713–1723.

57. Jones E, McGonagle D: **Human bone marrow mesenchymal stem cells in vivo**. *Rheumatol Oxf Engl* 2008, **47**:126–131.

58. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P: **Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment**. *Cell* 2007, **131**:324–336.

59. Delorme B, Ringe J, Gallay N, Le Vern Y, Kerboeuf D, Jorgensen C, Rosset P, Sensebé L, Layrolle P, Häupl T, Charbord P: **Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells**. *Blood* 2008, **111**:2631–2635.

60. Maijenburg MW, Kleijer M, Vermeul K, Mul EPJ, van Alphen FPJ, van der Schoot CE, Voermans C: **The composition of the mesenchymal stromal cell compartment in human bone marrow changes during development and aging**. *Haematologica* 2012, **97**:179–183.

61. Sousa BR, Parreira RC, Fonseca EA, Amaya MJ, Tonelli FMP, Lacerda SMSN, Lalwani P, Santos AK, Gomes KN, Ulrich H, Kihara AH, Resende RR: **Human adult stem cells from diverse origins: an overview from multiparametric immunophenotyping to clinical applications**. *Cytom Part J Int Soc Anal Cytol* 2014, **85**:43–77.

62. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T: **Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts**. *Cell* 1997, **89**:755–764.

63. Galotto M, Campanile G, Robino G, Cancedda FD, Bianco P, Cancedda R: **Hypertrophic chondrocytes undergo further differentiation to osteoblast-like cells and participate in the initial bone formation in developing chick embryo**. *J Bone Miner Res Off J Am Soc Bone Miner Res* 1994, **9**:1239–1249.

64. Gentili C, Bianco P, Neri M, Malpeli M, Campanile G, Castagnola P, Cancedda R, Cancedda FD: **Cell proliferation, extracellular matrix mineralization, and ovotransferrin transient expression during in vitro differentiation of chick hypertrophic chondrocytes into osteoblast-like cells**. *J Cell Biol* 1993, **122**:703–712.

65. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luriá EA, Ruadkow IA: **Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method**. *Exp Hematol* 1974, **2**:83–92.

66. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH: **Multilineage cells from human adipose tissue: implications for cell-based therapies**. *Tissue Eng* 2001, **7**:211–228.

67. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM: **Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT)**. *Cytotherapy* 2013, **15**:641–648.

68. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW: **Obesity is associated with macrophage accumulation in adipose tissue**. *J Clin Invest* 2003, **112**:1796–1808.

69. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: **Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance**. *J Clin Invest* 2003, **112**:1821–1830.

70. Mizuno H: **Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review**. *J Nippon Med Sch Nippon Ika Daigaku Zasshi* 2009, **76**:56–66.

71. Parker AM, Katz AJ: **Adipose-derived stem cells for the regeneration of damaged tissues**. *Expert Opin Biol Ther* 2006, **6**:567–578.

72. Fraser JK, Wulur I, Alfonso Z, Hedrick MH: **Fat tissue: an underappreciated source of stem cells for biotechnology**. *Trends Biotechnol* 2006, **24**:150–154.

73. Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, Jung JS: **Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue**. *Cell Physiol Biochem Int J Exp Cell Physiol Biochem Pharmacol* 2004, **14**:311–324.

74. Noël D, Caton D, Roche S, Bony C, Lehmann S, Casteilla L, Jorgensen C, Cousin B: **Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials**. *Exp Cell Res* 2008, **314**:1575–1584.

75. Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD: **Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood**. *Exp Hematol* 2005, **33**:1402–1416.

76. Jack GS, Almeida FG, Zhang R, Alfonso ZC, Zuk PA, Rodríguez LV: **Processed lipoaspirate cells for tissue engineering of the lower urinary tract: implications for the treatment of stress urinary incontinence and bladder reconstruction**. *J Urol* 2005, **174**:2041–2045.

77. Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH: **Myogenic differentiation by human processed lipoaspirate cells**. *Plast Reconstr Surg* 2002, **109**:199–209; discussion 210–211.

78. Lindroos B, Suuronen R, Miettinen S: **The potential of adipose stem cells in regenerative medicine**. *Stem Cell Rev* 2011, **7**:269–291.

79. Levi B, Longaker MT: **Concise review: adipose-derived stromal cells for skeletal regenerative medicine**. *Stem Cells Dayt Ohio* 2011, **29**:576–582.

80. Gargett CE, Schwab KE, Zillwood RM, Nguyen HPT, Wu D: **Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium**. *Biol Reprod* 2009, **80**:1136–1145.

81. Taha MF, Hedayati V: **Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells**. *Tissue Cell* 2010, **42**:211–216.

82. Schwab KE, Chan RWS, Gargett CE: **Putative stem cell activity of human endometrial epithelial and stromal cells during the menstrual cycle**. *Fertil Steril* 2005, **84 Suppl 2**:1124–1130.

83. Nakanishi C, Nagaya N, Ohnishi S, Yamahara K, Takabatake S, Konno T, Hayashi K, Kawashiri M-A, Tsubokawa T, Yamagishi M: **Gene and protein expression analysis of mesenchymal stem cells derived from rat adipose tissue and bone marrow**. *Circ J Off J Jpn Circ Soc* 2011, **75**:2260–2268.

84. Sng J, Lufkin T: **Emerging stem cell therapies: treatment, safety, and biology**. *Stem Cells Int* 2012, **2012**:521343.

85. Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI: **Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use**. *Bone Marrow Transplant* 1995, **16**:557–564.

86. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, Ringdén O: **Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells**. *Lancet* 2004, **363**:1439–1441.

87. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O, Developmental Committee of the European Group for Blood and Marrow Transplantation: **Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study**. *Lancet* 2008, **371**:1579–1586.

88. Sharma RR, Pollock K, Hubel A, McKenna D: **Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices**. *Transfusion (Paris)* 2014, **54**:1418–1437.

89. Baiguera S, Jungebluth P, Burns A, Mavilia C, Haag J, De Coppi P, Macchiarini P: **Tissue engineered human tracheas for in vivo implantation**. *Biomaterials* 2010, **31**:8931–8938.

90. Knoepfler PS: **Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine**. *Stem Cells Dayt Ohio* 2009, **27**:1050–1056.

91. Wakitani S, Okabe T, Horibe S, Mitsuoka T, Saito M, Koyama T, Nawata M, Tensho K, Kato H, Uematsu K, Kuroda R, Kurosaka M, Yoshiya S, Hattori K, Ohgushi H: **Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months**. *J Tissue Eng Regen Med* 2011, **5**:146–150.

92. Centeno CJ, Schultz JR, Cheever M, Freeman M, Faulkner S, Robinson B, Hanson R: **Safety and complications reporting update on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique**. *Curr Stem Cell Res Ther* 2011, **6**:368–378.

93. Varma HS, Dadarya B, Vidyarthi A: **The new avenues in the management of osteo-arthritis of knee--stem cells**. *J Indian Med Assoc* 2010, **108**:583–585.

94. Wakitani S, Nawata M, Tensho K, Okabe T, Machida H, Ohgushi H: **Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees**. *J Tissue Eng Regen Med* 2007, **1**:74–79.

95. Horwitz EM, Gordon PL, Koo WKK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T: **Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone**. *Proc Natl Acad Sci U S A* 2002, **99**:8932–8937.

96. Cowan CM, Shi Y-Y, Aalami OO, Chou Y-F, Mari C, Thomas R, Quarto N, Contag CH, Wu B, Longaker MT: **Adipose-derived adult stromal cells heal critical-size mouse calvarial defects**. *Nat Biotechnol* 2004, **22**:560–567.

97. Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt H-P: **Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report**. *J Cranio-Maxillo-fac Surg Off Publ Eur Assoc Cranio-Maxillo-fac Surg* 2004, **32**:370–373.

98. Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, Gerstenblith G, DeMaria AN, Denktas AE, Gammon RS, Hermiller JB, Reisman MA, Schaer GL, Sherman W: **A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction**. *J Am Coll Cardiol* 2009, **54**:2277–2286.

99. Mohyeddin-Bonab M, Mohamad-Hassani M-R, Alimoghaddam K, Sanatkar M, Gasemi M, Mirkhani H, Radmehr H, Salehi M, Eslami M, Farhig-Parsa A, Emami-Razavi H, Alemohammad MG, al-Mohamad MG, Solimani A-A, Ghavamzadeh A, Nikbin B: **Autologous in vitro expanded mesenchymal stem cell therapy for human old myocardial infarction**. *Arch Iran Med* 2007, **10**:467–473.

100. Mazo M, Araña M, Pelacho B, Prosper F: **Mesenchymal stem cells and cardiovascular disease: a bench to bedside roadmap**. *Stem Cells Int* 2012, **2012**:175979.

101. Agorogiannis GI, Alexaki V-I, Castana O, Kymionis GD: **Topical application of autologous adipose-derived mesenchymal stem cells (MSCs) for persistent sterile corneal epithelial defect**. *Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Für Klin Exp Ophthalmol* 2012, **250**:455–457.

102. García-Olmo D, García-Arranz M, García LG, Cuellar ES, Blanco IF, Prianes LA, Montes JAR, Pinto FL, Marcos DH, García-Sancho L: **Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy**. *Int J Colorectal Dis* 2003, **18**:451–454.

103. García-Olmo D, García-Arranz M, Herreros D, Pascual I, Peiro C, Rodríguez-Montes JA: **A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation**. *Dis Colon Rectum* 2005, **48**:1416–1423.

104. Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M: **Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial**. *Dis Colon Rectum* 2009, **52**:79–86.

105. Ankrum J, Karp JM: **Mesenchymal stem cell therapy: Two steps forward, one step back**. *Trends Mol Med* 2010, **16**:203–209.

106. Plotnikov EY, Khryapenkova TG, Vasileva AK, Marey MV, Galkina SI, Isaev NK, Sheval EV, Polyakov VY, Sukhikh GT, Zorov DB: **Cell-to-cell cross-talk between mesenchymal stem cells and cardiomyocytes in co-culture**. *J Cell Mol Med* 2008, **12**:1622–1631.

107. Cselenyák A, Pankotai E, Horváth EM, Kiss L, Lacza Z: **Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cell-to-cell connections**. *BMC Cell Biol* 2010, **11**:29.

108. Law RHP, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI, Whisstock JC: **An overview of the serpin superfamily**. *Genome Biol* 2006, **7**:216.

109. Kozaki K, Miyaishi O, Koiwai O, Yasui Y, Kashiwai A, Nishikawa Y, Shimizu S, Saga S: **Isolation, purification, and characterization of a collagen-associated serpin, caspin, produced by murine colon adenocarcinoma cells**. *J Biol Chem* 1998, **273**:15125–15130.

110. Van Gent D, Sharp P, Morgan K, Kalsheker N: **Serpins: structure, function and molecular evolution**. *Int J Biochem Cell Biol* 2003, **35**:1536–1547.

111. Carrell R, Lomas D, Stein P, Whisstock J: **Dysfunctional variants and the structural biology of the serpins**. *Adv Exp Med Biol* 1997, **425**:207–222.

112. Tombran-Tink J: **The neuroprotective and angiogenesis inhibitory serpin, PEDF: new insights into phylogeny, function, and signaling**. *Front Biosci J Virtual Libr* 2005, **10**:2131–2149.

113. Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC: **The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature**. *J Biol Chem* 2001, **276**:33293–33296.

114. Tombran-Tink J, Johnson LV: **Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells**. *Invest Ophthalmol Vis Sci* 1989, **30**:1700–1707.

115. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J: **Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family**. *Proc Natl Acad Sci U S A* 1993, **90**:1526–1530.

116. Tombran-Tink J, Aparicio S, Xu X, Tink AR, Lara N, Sawant S, Barnstable CJ, Zhang SS-M: **PEDF and the serpins: Phylogeny, sequence conservation, and functional domains**. *J Struct Biol* 2005, **151**:130–150.

117. Alberdi E, Aymerich MS, Becerra SP: **Binding of pigment epithelium-derived factor (PEDF) to retinoblastoma cells and cerebellar granule neurons. Evidence for a PEDF receptor**. *J Biol Chem* 1999, **274**:31605–31612.

118. Becerra SP, Sagasti A, Spinella P, Notario V: **Pigment epithelium-derived factor behaves like a noninhibitory serpin. Neurotrophic activity does not require the serpin reactive loop**. *J Biol Chem* 1995, **270**:25992–25999.

119. Tombran-Tink J, Mazuruk K, Rodriguez IR, Chung D, Linker T, Englander E, Chader GJ: **Organization, evolutionary conservation, expression and unusual Alu density of the human gene for pigment epithelium-derived factor, a unique neurotrophic serpin**. *Mol Vis* 1996, **2**:11.

120. Xu X, Zhang SS-M, Barnstable CJ, Tombran-Tink J: **Molecular phylogeny of the antiangiogenic and neurotrophic serpin, pigment epithelium derived factor in vertebrates**. *BMC Genomics* 2006, **7**:248.

121. Simonovic M, Gettins PG, Volz K: **Crystal structure of human PEDF, a potent anti-angiogenic and neurite growth-promoting factor**. *Proc Natl Acad Sci U S A* 2001, **98**:11131–11135.

122. Alberdi E, Hyde CC, Becerra SP: **Pigment epithelium-derived factor (PEDF) binds to glycosaminoglycans: analysis of the binding site**. *Biochemistry (Mosc)* 1998, **37**:10643–10652.

123. Stratikos E, Alberdi E, Gettins PG, Becerra SP: **Recombinant human pigment epithelium-derived factor (PEDF): characterization of PEDF overexpressed and secreted by eukaryotic cells**. *Protein Sci Publ Protein Soc* 1996, **5**:2575–2582.

124. Chader GJ: **PEDF: Raising both hopes and questions in controlling angiogenesis**. *Proc Natl Acad Sci U S A* 2001, **98**:2122–2124.

125. Chandolu V, Dass CR: **Cell and Molecular Biology Underpinning the Effects of PEDF on Cancers in General and Osteosarcoma in Particular**. *J Biomed Biotechnol* 2012, **2012**:1–9.

126. Meyer C: **Mapping the Type I Collagen-binding Site on Pigment Epithelium-derived Factor. IMPLICATIONS FOR ITS ANTIANGIOGENIC ACTIVITY**. *J Biol Chem* 2002, **277**:45400–45407.

127. Yasui N, Mori T, Morito D, Matsushita O, Kourai H, Nagata K, Koide T: **Dual-Site Recognition of Different Extracellular Matrix Components by Anti-Angiogenic/Neurotrophic Serpin, PEDF**. *Biochemistry (Mosc)* 2003, **42**:3160–3167.

128. Becerra SP, Perez-Mediavilla LA, Weldon JE, Locatelli-Hoops S, Senanayake P, Notari L, Notario V, Hollyfield JG: **Pigment epithelium-derived factor binds to hyaluronan. Mapping of a hyaluronan binding site**. *J Biol Chem* 2008, **283**:33310–33320.

129. Maik-Rachline G, Seger R: **Variable phosphorylation states of pigment-epithelium-derived factor differentially regulate its function**. *Blood* 2006, **107**:2745–2752.

130. Ek ETH, Dass CR, Choong PFM: **PEDF: a potential molecular therapeutic target with multiple anti-cancer activities**. *Trends Mol Med* 2006, **12**:497–502.

131. Filleur S, Nelius T, de Riese W, Kennedy RC: **Characterization of PEDF: A multi-functional serpin family protein**. *J Cell Biochem* 2009, **106**:769–775.

132. Aymerich MS, Alberdi EM, Martínez A, Becerra SP: **Evidence for pigment epithelium-derived factor receptors in the neural retina**. *Invest Ophthalmol Vis Sci* 2001, **42**:3287–3293.

133. Barnstable CJ, Tombran-Tink J: **Neuroprotective and antiangiogenic actions of PEDF in the eye: molecular targets and therapeutic potential**. *Prog Retin Eye Res* 2004, **23**:561–577.

134. Petersen S, Valnickova Z, Enghild J: **Pigment-epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization**. *Biochem J* 2003, **374**:199–206.

135. Costacou T, Zgibor JC, Evans RW, Otvos J, Lopes-Virella MF, Tracy RP, Orchard TJ: **The prospective association between adiponectin and coronary artery disease among individuals with type 1 diabetes. The Pittsburgh Epidemiology of Diabetes Complications Study**. *Diabetologia* 2005, **48**:41–48.

136. Famulla S, Lamers D, Hartwig S, Passlack W, Horrighs A, Cramer A, Lehr S, Sell H, Eckel J: **Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells**. *Int J Obes* 2010, **35**:762–772.

137. Moreno-Navarrete JM, Touskova V, Sabater M, Mraz M, Drapalova J, Ortega F, Serrano M, Catalán V, Gómez-Ambrosi J, Ortiz MR, Pardo G, Pueyo N, Ricart W, Lacinova Z, Haluzik M, Frühbeck G, Fernández-Real JM: **Liver, but not adipose tissue PEDF gene expression is associated with insulin resistance**. *Int J Obes* 2013, **37**:1230–1237.

138. Sawant S, Aparicio S, Tink AR, Lara N, Barnstable CJ, Tombran-Tink J: **Regulation of factors controlling angiogenesis in liver development: a role for PEDF in the formation and maintenance of normal vasculature**. *Biochem Biophys Res Commun* 2004, **325**:408–413.

139. Wang JJ, Zhang SX, Lu K, Chen Y, Mott R, Sato S, Ma J-X: **Decreased expression of pigment epithelium-derived factor is involved in the pathogenesis of diabetic nephropathy**. *Diabetes* 2005, **54**:243–250.

140. Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakao K, Eguchi K: **Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma**. *Hepatology* 2004, **40**:252–259.

141. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A: **A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes**. *Mol Cell Proteomics MCP* 2002, **1**:213–222.

142. Tombran-Tink J, Chader GG, Johnson LV: **PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity**. *Exp Eye Res* 1991, **53**:411–414.

143. Becerra SP: **Structure-function studies on PEDF. A noninhibitory serpin with neurotrophic activity**. *Adv Exp Med Biol* 1997, **425**:223–237.

144. Taniwaki T, Becerra SP, Chader GJ, Schwartz JP: **Pigment epithelium-derived factor is a survival factor for cerebellar granule cells in culture**. *J Neurochem* 1995, **64**:2509–2517.

145. Taniwaki T, Hirashima N, Becerra SP, Chader GJ, Etcheberrigaray R, Schwartz JP: **Pigment epithelium-derived factor protects cultured cerebellar granule cells against glutamate-induced neurotoxicity**. *J Neurochem* 1997, **68**:26–32.

146. Araki T, Taniwaki T, Becerra SP, Chader GJ, Schwartz JP: **Pigment epithelium-derived factor (PEDF) differentially protects immature but not mature cerebellar granule cells against apoptotic cell death**. *J Neurosci Res* 1998, **53**:7–15.

147. Cayouette M, Smith SB, Becerra SP, Gravel C: **Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations**. *Neurobiol Dis* 1999, **6**:523–532.

148. Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP: **Pigment epithelium-derived factor: a potent inhibitor of angiogenesis**. *Science* 1999, **285**:245–248.

149. Tonnesen MG, Feng X, Clark RA: **Angiogenesis in wound healing**. *J Investig Dermatol Symp Proc Soc Investig Dermatol Inc Eur Soc Dermatol Res* 2000, **5**:40–46.

150. Rychli K, Huber K, Wojta J: **Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease**. *Expert Opin Ther Targets* 2009, **13**:1295–1302.

151. Pignolo RJ, Cristofalo VJ, Rotenberg MO: **Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state**. *J Biol Chem* 1993, **268**:8949–8957.

152. Hjelmeland LM, Cristofolo VJ, Funk W, Rakoczy E, Katz ML: **Senescence of the retinal pigment epithelium**. *Mol Vis* 1999, **5**:33.

153. Pignolo RJ, Francis MK, Rotenberg MO, Cristofalo VJ: **Putative role for EPC-1/PEDF in the G0 growth arrest of human diploid fibroblasts**. *J Cell Physiol* 2003, **195**:12–20.

154. Palmieri D, Watson JM, Rinehart CA: **Age-related expression of PEDF/EPC-1 in human endometrial stromal fibroblasts: implications for interactive senescence**. *Exp Cell Res* 1999, **247**:142–147.

155. Gao X, Zhang H, Zhuang W, Yuan G, Sun T, Jiang X, Zhou Z, Yuan H, Zhang Z, Dong H: **PEDF and PEDF-derived peptide 44mer protect cardiomyocytes against hypoxia-induced apoptosis and necroptosis via anti-oxidative effect**. *Sci Rep* 2014, **4**:5637.

156. Quan GMY, Ojaimi J, Li Y, Kartsogiannis V, Zhou H, Choong PFM: **Localization of Pigment Epithelium-Derived Factor in Growing Mouse Bone**. *Calcif Tissue Int* 2005, **76**:146–153.

157. Lord EA, Martin SK, Gray JP, Li C, Clark DE: **Cell Cycle Genes PEDF and CDKN1C in Growing Deer Antlers**. *Anat Rec Adv Integr Anat Evol Biol* 2007, **290**:994–1004.

158. Crawford SE, Stellmach V, Ranalli M, Huang X, Huang L, Volpert O, De Vries GH, Abramson LP, Bouck N: **Pigment epithelium-derived factor (PEDF) in neuroblastoma: a multifunctional mediator of Schwann cell antitumor activity**. *J Cell Sci* 2001, **114**:4421–4428.

159. Wågsäter D, Löfgren S, Zar N, Hugander A, Dimberg J: **Pigment Epithelium-Derived Factor Expression in Colorectal Cancer Patients**. *Cancer Invest* 2010, **28**:872–877.

160. Lecke SB, Morsch D, Spritzer PM: **Circulating levels and subcutaneous adipose tissue gene expression of pigment epithelium-derived factor in polycystic ovary syndrome and normal women: a case control study**. *Reprod Biol Endocrinol RBE* 2013, **11**:77.

161. Liu J-T, Chen Y-L, Chen W-C, Chen H-Y, Lin Y-W, Wang S-H, Man K-M, Wan H-M, Yin W-H, Liu P-L, Chen Y-H: **Role of Pigment Epithelium-Derived Factor in Stem/Progenitor Cell-Associated Neovascularization**. *J Biomed Biotechnol* 2012, **2012**:1–10.

162. Notari L, Baladron V, Aroca-Aguilar JD, Balko N, Heredia R, Meyer C, Notario PM, Saravanamuthu S, Nueda M-L, Sanchez-Sanchez F, Escribano J, Laborda J, Becerra SP: **Identification of a Lipase-linked Cell Membrane Receptor for Pigment Epithelium-derived Factor**. *J Biol Chem* 2006, **281**:38022–38037.

163. Manalo KB, Choong PF, Becerra SP, Dass CR: **Pigment epithelium-derived factor as an anticancer drug and new treatment methods following the discovery of its receptors: a patent perspective**. *Expert Opin Ther Pat* 2011, **21**:121–130.

164. Filleur S, Volz K, Nelius T, Mirochnik Y, Huang H, Zaichuk TA, Aymerich MS, Becerra SP, Yap R, Veliceasa D, Shroff EH, Volpert OV: **Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer**. *Cancer Res* 2005, **65**:5144–5152.

165. Borg ML, Andrews ZB, Duh EJ, Zechner R, Meikle PJ, Watt MJ: **Pigment Epithelium-Derived Factor Regulates Lipid Metabolism via Adipose Triglyceride Lipase**. *Diabetes* 2011, **60**:1458–1466.

166. Chung C, Doll JA, Gattu AK, Shugrue C, Cornwell M, Fitchev P, Crawford SE: **Anti-angiogenic pigment epithelium-derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL)**. *J Hepatol* 2008, **48**:471–478.

167. Bernard A, Gao-Li J, Franco C-A, Bouceba T, Huet A, Li Z: **Laminin Receptor Involvement in the Anti-angiogenic Activity of Pigment Epithelium-derived Factor**. *J Biol Chem* 2009, **284**:10480–10490.

168. Notari L, Arakaki N, Mueller D, Meier S, Amaral J, Becerra SP: **Pigment epithelium-derived factor binds to cell-surface F(1)-ATP synthase**. *FEBS J* 2010, **277**:2192–2205.

169. Tombran-Tink J, Barnstable CJ: **Therapeutic prospects for PEDF: more than a promising angiogenesis inhibitor**. *Trends Mol Med* 2003, **9**:244–250.

170. Thumann G: **Prospectives for gene therapy of retinal degenerations**. *Curr Genomics* 2012, **13**:350.

171. Kanda S, Mochizuki Y, Nakamura T, Miyata Y, Matsuyama T, Kanetake H: **Pigment epithelium-derived factor inhibits fibroblast-growth-factor-2-induced capillary morphogenesis of endothelial cells through Fyn**. *J Cell Sci* 2005, **118**(Pt 5):961–970.

172. Broadhead ML, Dass CR, Choong PFM: **In vitro and in vivo biological activity of PEDF against a range of tumors**. *Expert Opin Ther Targets* 2009, **13**:1429–1438.

173. Benjamin LE: **The controls of microvascular survival**. *Cancer Metastasis Rev* 2000, **19**:75–81.

174. Wang L, Schmitz V, Perez-Mediavilla A, Izal I, Prieto J, Qian C: **Suppression of angiogenesis and tumor growth by adenoviral-mediated gene transfer of pigment epithelium-derived factor**. *Mol Ther J Am Soc Gene Ther* 2003, **8**:72–79.

175. Chen L, Zhang SS-M, Barnstable CJ, Tombran-Tink J: **PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases**. *Biochem Biophys Res Commun* 2006, **348**:1288–1295.

176. Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP: **Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor**. *Nat Med* 2002, **8**:349–357.

177. Ablonczy Z, Prakasam A, Fant J, Fauq A, Crosson C, Sambamurti K: **Pigment epithelium-derived factor maintains retinal pigment epithelium function by inhibiting vascular endothelial growth factor-R2 signaling through gamma-secretase**. *J Biol Chem* 2009, **284**:30177–30186.

178. Cai J, Jiang WG, Grant MB, Boulton M: **Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1**. *J Biol Chem* 2006, **281**:3604–3613.

179. Cai J, Chen Z, Ruan Q, Han S, Liu L, Qi X, Boye SL, Hauswirth WW, Grant MB, Boulton ME: **γ-Secretase and presenilin mediate cleavage and phosphorylation of vascular endothelial growth factor receptor-1**. *J Biol Chem* 2011, **286**:42514–42523.

180. Konson A, Pradeep S, D'Acunto CW, Seger R: **Pigment Epithelium-derived Factor and Its Phosphomimetic Mutant Induce JNK-dependent Apoptosis and p38-mediated Migration Arrest**. *J Biol Chem* 2011, **286**:3540–3551.

181. Ho T-C, Chen S-L, Shih S-C, Chang S-J, Yang S-L, Hsieh J-W, Cheng H-C, Chen L-J, Tsao Y-P: **Pigment epithelium-derived factor (PEDF) promotes tumor cell death by inducing macrophage membrane tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)**. *J Biol Chem* 2011, **286**:35943–35954.

182. Biyashev D, Veliceasa D, Kwiatek A, Sutanto MM, Cohen RN, Volpert OV: **Natural angiogenesis inhibitor signals through Erk5 activation of peroxisome proliferator-activated receptor gamma (PPARgamma)**. *J Biol Chem* 2010, **285**:13517–13524.

183. Abramson LP, Stellmach V, Doll JA, Cornwell M, Arensman RM, Crawford SE: **Wilms' tumor growth is suppressed by antiangiogenic pigment epithelium-derived factor in a xenograft model**. *J Pediatr Surg* 2003, **38**:336–342; discussion 336–342.

184. Doll JA, Stellmach VM, Bouck NP, Bergh ARJ, Lee C, Abramson LP, Cornwell ML, Pins MR, Borensztajn J, Crawford SE: **Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas**. *Nat Med* 2003, **9**:774–780.

185. Seth P: **Vector-mediated cancer gene therapy: an overview**. *Cancer Biol Ther* 2005, **4**:512–517.

186. Davidoff AM, Leary MA, Ng CYC, Vanin EF: **Gene therapy-mediated expression by tumor cells of the angiogenesis inhibitor flk-1 results in inhibition of neuroblastoma growth in vivo**. *J Pediatr Surg* 2001, **36**:30–36.

187. Manley PW, Martiny-Baron G, Schlaeppi J-M, Wood JM: **Therapies directed at vascular endothelial growth factor**. *Expert Opin Investig Drugs* 2002, **11**:1715–1736.

188. Rosen LS: **Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers**. *Cancer Control J Moffitt Cancer Cent* 2002, **9**(2 Suppl):36–44.

189. Bouck N: **PEDF: anti-angiogenic guardian of ocular function**. *Trends Mol Med* 2002, **8**:330–334.

190. Abe R, Shimizu T, Yamagishi S, Shibaki A, Amano S, Inagaki Y, Watanabe H, Sugawara H, Nakamura H, Takeuchi M, others: **Overexpression of Pigment Epithelium-Derived Factor Decreases Angiogenesis and Inhibits the Growth of Human Malignant Melanoma Cells in Vivo**. *Am J Pathol* 2004, **164**:1225–1232.

191. Takenaka K, Yamagishi S, Jinnouchi Y, Nakamura K, Matsui T, Imaizumi T: **Pigment epithelium-derived factor (PEDF)-induced apoptosis and inhibition of vascular endothelial growth factor (VEGF) expression in MG63 human osteosarcoma cells**. *Life Sci* 2005, **77**:3231–3241.

192. Ek ETH, Dass CR, Contreras KG, Choong PFM: **Inhibition of orthotopic osteosarcoma growth and metastasis by multitargeted antitumor activities of pigment epithelium-derived factor**. *Clin Exp Metastasis* 2007, **24**:93–106.

193. Ek ETH, Dass CR, Contreras KG, Choong PFM: **PEDF-derived synthetic peptides exhibit antitumor activity in an orthotopic model of human osteosarcoma**. *J Orthop Res* 2007, **25**:1671–1680.

194. Ning T, Yan X, Lu Z-J, Wang G-P, Zhang N-G, Yang J-L, Jiang S-S, Wu Y, Yang L, Guan Y-S, Luo F: **Gene therapy with the angiogenesis inhibitor endostatin in an orthotopic lung cancer murine model**. *Hum Gene Ther* 2009, **20**:103–111.

195. Liu X, Chen H-H, Zhang L-W: **Potential therapeutic effects of pigment epithelium-derived factor for treatment of diabetic retinopathy**. *Int J Ophthalmol* 2013, **6**:221–227.

196. Garcia-Ramirez M, Toran N, Andaluz P, Carrascosa A, Audi L: **Vascular endothelial growth factor is expressed in human fetal growth cartilage**. *J Bone Miner Res Off J Am Soc Bone Miner Res* 2000, **15**:534–540.

197. Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE: **Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage**. *J Anat* 1999, **194 (Pt 4)**:519–524.

198. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N: **VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation**. *Nat Med* 1999, **5**:623–628.

199. Quan GMY, Ojaimi J, Nadesapillai APW, Zhou H, Choong PFM: **Resistance of Epiphyseal Cartilage to Invasion by Osteosarcoma Is Likely to Be Due to Expression of Antiangiogenic Factors**. *Pathobiology* 2002, **70**:361–367.

200. Tombran-Tink J, Barnstable C.: **Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone**. *Biochem Biophys Res Commun* 2004, **316**:573–579.

201. Venturi G, Gandini A, Monti E, Carbonare LD, Corradi M, Vincenzi M, Valenti MT, Valli M, Pelilli E, Boner A, Mottes M, Antoniazzi F: **Lack of expression of *SERPINF1* , the gene coding for pigment epithelium-derived factor, causes progressively deforming osteogenesis imperfecta with normal type I collagen**. *J Bone Miner Res* 2012, **27**:723–728.

202. Rauch F, Glorieux FH: **Osteogenesis imperfecta**. *Lancet* 2004, **363**:1377–1385.

203. Phillipi CA, Remmington T, Steiner RD: **Bisphosphonate therapy for osteogenesis imperfecta**. *Cochrane Database Syst Rev* 2008:CD005088.

204. Glorieux FH, Ward LM, Rauch F, Lalic L, Roughley PJ, Travers R: **Osteogenesis imperfecta type VI: a form of brittle bone disease with a mineralization defect**. *J Bone Miner Res Off J Am Soc Bone Miner Res* 2002, **17**:30–38.

205. Land C, Rauch F, Travers R, Glorieux FH: **Osteogenesis imperfecta type VI in childhood and adolescence: effects of cyclical intravenous pamidronate treatment**. *Bone* 2007, **40**:638–644.

206. Bogan R, Riddle RC, Li Z, Kumar S, Nandal A, Faugere M-C, Boskey A, Crawford SE, Clemens TL: **A mouse model for human osteogenesis imperfecta type VI**. *J Bone Miner Res Off J Am Soc Bone Miner Res* 2013, **28**:1531–1536.

207. Al-Jallad H, Palomo T, Moffatt P, Roughley P, Glorieux FH, Rauch F: **Normal Bone Density and Fat Mass in Heterozygous *SERPINF1* Mutation Carriers**. *J Clin Endocrinol Metab* 2014:jc.2014–2505.

208. Rauch F, Husseini A, Roughley P, Glorieux FH, Moffatt P: **Lack of Circulating Pigment Epithelium-Derived Factor Is a Marker of Osteogenesis Imperfecta Type VI**. *J Clin Endocrinol Metab* 2012, **97**:E1550–E1556.

209. Fratzl-Zelman N, Schmidt I, Roschger P, Roschger A, Glorieux FH, Klaushofer K, Wagermaier W, Rauch F, Fratzl P: **Unique micro- and nano-scale mineralization pattern of human osteogenesis imperfecta type VI bone**. *Bone* 2014, **73C**:233–241.

210. Puchtler H, Meloan SN: **Demonstration of phosphates in calcium deposits: a modification of von Kossa's reaction**. *Histochemistry* 1978, **56**:177–185.

211. Veronesi E, Murgia A, Caselli A, Grisendi G, Piccinno MS, Rasini V, Giordano R, Montemurro T, Bourin P, Sensebé L, Rojewski MT, Schrezenmeier H, Layrolle P, Ginebra MP, Panaitescu CB, Gómez-Barrena E, Catani F, Paolucci P, Burns JS, Dominici M: **Transportation conditions for prompt use of ex vivo expanded and freshly harvested clinical-grade bone marrow mesenchymal stromal/stem cells for bone regeneration**. *Tissue Eng Part C Methods* 2014, **20**:239–251.

212. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH: **Multilineage cells from human adipose tissue: implications for cell-based therapies**. *Tissue Eng* 2001, **7**:211–228.

213. Traut W, Endl E, Scholzen T, Gerdes J, Winking H: **The temporal and spatial distribution of the proliferation associated Ki-67 protein during female and male meiosis**. *Chromosoma* 2002, **111**:156–164.

214. Brown DC, Gatter KC: **Monoclonal antibody Ki-67: its use in histopathology**. *Histopathology* 1990, **17**:489–503.

215. Anderson WF: **Human gene therapy**. *Science* 1992, **256**:808–813.

216. D'souza N, Burns JS, Grisendi G, Candini O, Veronesi E, Piccinno S, Horwitz EM, Paolucci P, Conte P, Dominici M: **MSC and Tumors: Homing, Differentiation, and Secretion Influence Therapeutic Potential**. *Adv Biochem Eng Biotechnol* 2013, **130**:209–266.

217. Porada CD, Almeida-Porada G: **Mesenchymal stem cells as therapeutics and vehicles for gene and drug delivery**. *Adv Drug Deliv Rev* 2010, **62**:1156–1166.

218. Fillat C, Carrió M, Cascante A, Sangro B: **Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application**. *Curr Gene Ther* 2003, **3**:13–26.

219. Secchiero P, Melloni E, Corallini F, Beltrami AP, Alviano F, Milani D, D'Aurizio F, di Iasio MG, Cesselli D, Bagnara GP, Zauli G: **Tumor necrosis factor-related apoptosis-inducing ligand promotes migration of human bone marrow multipotent stromal cells**. *Stem Cells Dayt Ohio* 2008, **26**:2955–2963.

220. Allay JA, Dennis JE, Haynesworth SE, Majumdar MK, Clapp DW, Shultz LD, Caplan AI, Gerson SL: **LacZ and interleukin-3 expression in vivo after retroviral transduction of marrow-derived human osteogenic mesenchymal progenitors**. *Hum Gene Ther* 1997, **8**:1417–1427.

221. Matthews KE, Keating A: **Gene therapy with physical methods of gene transfer**. *Transfus Sci* 1996, **17**:29–34.

222. Koç ON, Lazarus HM: **Mesenchymal stem cells: heading into the clinic**. *Bone Marrow Transplant* 2001, **27**:235–239.

223. Chang SC-N, Chuang HL, Chen YR, Chen JK, Chung H-Y, Lu Y-L, Lin H-Y, Tai C-L, Lou J: **Ex vivo gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration**. *Gene Ther* 2003, **10**:2013–2019.

224. Gugala Z, Olmsted-Davis EA, Gannon FH, Lindsey RW, Davis AR: **Osteoinduction by ex vivo adenovirus-mediated BMP2 delivery is independent of cell type**. *Gene Ther* 2003, **10**:1289–1296.

225. Zhang XS, Linkhart TA, Chen S-T, Peng H, Wergedal JE, Guttierez GG, Sheng MH-C, Lau K-HW, Baylink DJ: **Local ex vivo gene therapy with bone marrow stromal cells expressing human BMP4 promotes endosteal bone formation in mice**. *J Gene Med* 2004, **6**:4–15.

226. Choi J-J, Yoo S-A, Park S-J, Kang Y-J, Kim W-U, Oh I-H, Cho C-S: **Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice**. *Clin Exp Immunol* 2008, **153**:269–276.

227. Kumar S, Nagy TR, Ponnazhagan S: **Therapeutic potential of genetically modified adult stem cells for osteopenia**. *Gene Ther* 2010, **17**:105–116.

228. Sasaki M, Radtke C, Tan AM, Zhao P, Hamada H, Houkin K, Honmou O, Kocsis JD: **BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury**. *J Neurosci Off J Soc Neurosci* 2009, **29**:14932–14941.

229. Karnieli O, Izhar-Prato Y, Bulvik S, Efrat S: **Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation**. *Stem Cells Dayt Ohio* 2007, **25**:2837–2844.

230. Li Y, Zhang R, Qiao H, Zhang H, Wang Y, Yuan H, Liu Q, Liu D, Chen L, Pei X: **Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells**. *J Cell Physiol* 2007, **211**:36–44.

231. Passier R, van Laake LW, Mummery CL: **Stem-cell-based therapy and lessons from the heart**. *Nature* 2008, **453**:322–329.

232. Mirotsou M, Zhang Z, Deb A, Zhang L, Gnecchi M, Noiseux N, Mu H, Pachori A, Dzau V: **Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair**. *Proc Natl Acad Sci U S A* 2007, **104**:1643–1648.

233. Tang YL, Tang Y, Zhang YC, Qian K, Shen L, Phillips MI: **Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector**. *J Am Coll Cardiol* 2005, **46**:1339–1350.

234. Li W, Ma N, Ong L-L, Nesselmann C, Klopsch C, Ladilov Y, Furlani D, Piechaczek C, Moebius JM, Lützow K, Lendlein A, Stamm C, Li R-K, Steinhoff G: **Bcl-2 engineered MSCs inhibited apoptosis and improved heart function**. *Stem Cells Dayt Ohio* 2007, **25**:2118–2127.

235. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ: **Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts**. *Nat Med* 2003, **9**:1195–1201.

236. Klinge PM, Harmening K, Miller MC, Heile A, Wallrapp C, Geigle P, Brinker T: **Encapsulated native and glucagon-like peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease**. *Neurosci Lett* 2011, **497**:6–10.

237. Song YS, Lee HJ, Doo SH, Lee SJ, Lim I, Chang K-T, Kim SU: **Mesenchymal stem cells overexpressing hepatocyte growth factor (HGF) inhibit collagen deposit and improve bladder function in rat model of bladder outlet obstruction**. *Cell Transplant* 2012, **21**:1641–1650.

238. Aboody KS, Najbauer J, Danks MK: **Stem and progenitor cell-mediated tumor selective gene therapy**. *Gene Ther* 2008, **15**:739–752.

239. Nakamura K, Ito Y, Kawano Y, Kurozumi K, Kobune M, Tsuda H, Bizen A, Honmou O, Niitsu Y, Hamada H: **Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model**. *Gene Ther* 2004, **11**:1155–1164.

240. Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, Chen J, Hentschel S, Vecil G, Dembinski J, Andreeff M, Lang FF: **Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas**. *Cancer Res* 2005, **65**:3307–3318.

241. Ren C, Kumar S, Chanda D, Chen J, Mountz JD, Ponnazhagan S: **Therapeutic potential of mesenchymal stem cells producing interferon-alpha in a mouse melanoma lung metastasis model**. *Stem Cells Dayt Ohio* 2008, **26**:2332–2338.

242. Xin H, Kanehira M, Mizuguchi H, Hayakawa T, Kikuchi T, Nukiwa T, Saijo Y: **Targeted delivery of CX3CL1 to multiple lung tumors by mesenchymal stem cells**. *Stem Cells Dayt Ohio* 2007, **25**:1618–1626.

243. Grisendi G, Bussolari R, Cafarelli L, Petak I, Rasini V, Veronesi E, De Santis G, Spano C, Tagliazzucchi M, Barti-Juhasz H, Scarabelli L, Bambi F, Frassoldati A, Rossi G, Casali C, Morandi U, Horwitz EM, Paolucci P, Conte P, Dominici M: **Adipose-derived mesenchymal stem cells as stable source of tumor necrosis factor-related apoptosis-inducing ligand delivery for cancer therapy**. *Cancer Res* 2010, **70**:3718–3729.

244. Grisendi G, Spano C, D'souza N, Rasini V, Veronesi E, Prapa M, Petrachi T, Piccinno S, Rossignoli F, Burns JS, Fiorcari S, Granchi D, Baldini N, Horwitz EM, Guarneri V, Conte P, Paolucci P, Dominici M: **Mesenchymal Progenitors Expressing TRAIL Induce Apoptosis in Sarcomas**. *Stem Cells Dayt Ohio* 2015, **33**:859–869.

245. Loebinger MR, Eddaoudi A, Davies D, Janes SM: **Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer**. *Cancer Res* 2009, **69**:4134–4142.

246. Menon LG, Kelly K, Yang HW, Kim S-K, Black PM, Carroll RS: **Human bone marrow-derived mesenchymal stromal cells expressing S-TRAIL as a cellular delivery vehicle for human glioma therapy**. *Stem Cells Dayt Ohio* 2009, **27**:2320–2330.

247. Sasportas LS, Kasmieh R, Wakimoto H, Hingtgen S, van de Water JAJM, Mohapatra G, Figueiredo JL, Martuza RL, Weissleder R, Shah K: **Assessment of therapeutic efficacy and fate of engineered human mesenchymal stem cells for cancer therapy**. *Proc Natl Acad Sci U S A* 2009, **106**:4822–4827.

248. Miletic H, Fischer YH, Giroglou T, Rueger MA, Winkeler A, Li H, Himmelreich U, Stenzel W, Jacobs AH, von Laer D: **Normal brain cells contribute to the bystander effect in suicide gene therapy of malignant glioma**. *Clin Cancer Res Off J Am Assoc Cancer Res* 2007, **13**(22 Pt 1):6761–6768.

249. Danks MK, Yoon KJ, Bush RA, Remack JS, Wierdl M, Tsurkan L, Kim SU, Garcia E, Metz MZ, Najbauer J, Potter PM, Aboody KS: **Tumor-targeted enzyme/prodrug therapy mediates long-term disease-free survival of mice bearing disseminated neuroblastoma**. *Cancer Res* 2007, **67**:22–25.

250. Kanehira M, Xin H, Hoshino K, Maemondo M, Mizuguchi H, Hayakawa T, Matsumoto K, Nakamura T, Nukiwa T, Saijo Y: **Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells**. *Cancer Gene Ther* 2007, **14**:894–903.

251. Komarova S, Kawakami Y, Stoff-Khalili MA, Curiel DT, Pereboeva L: **Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses**. *Mol Cancer Ther* 2006, **5**:755–766.

252. Numnum TM, Makhija S, Lu B, Wang M, Rivera A, Stoff-Khalili M, Alvarez RD, Zhu ZB, Curiel DT: **Improved anti-tumor therapy based upon infectivity-enhanced adenoviral delivery of RNA interference in ovarian carcinoma cell lines**. *Gynecol Oncol* 2008, **108**:34–41.

253. Ek ETH, Dass CR, Choong PFM: **Pigment epithelium-derived factor: a multimodal tumor inhibitor**. *Mol Cancer Ther* 2006, **5**:1641–1646.

254. Wei L: **Adenovector pigment epithelium-derived factor (AdPEDF) delivery for wet age-related macular degeneration**. *Retina Phila Pa* 2005, **25**(8 Suppl):S48–S49.

255. Arnhold S, Heiduschka P, Klein H, Absenger Y, Basnaoglu S, Kreppel F, Henke-Fahle S, Kochanek S, Bartz-Schmidt KU, Addicks K, Schraermeyer U.: **Adenovirally Transduced Bone Marrow Stromal Cells Differentiate into Pigment Epithelial Cells and Induce Rescue Effects in RCS Rats**. *Invest Ophthalmol Vis Sci* 2006, **47**:4121–4129.

256. Hou H-Y, Liang H-L, Wang Y-S, Zhang Z-X, Wang B-R, Shi Y-Y, Dong X, Cai Y: **A Therapeutic Strategy for Choroidal Neovascularization Based on Recruitment of Mesenchymal Stem Cells to the Sites of Lesions**. *Mol Ther* 2010, **18**:1837–1845.

257. Mori K, Gehlbach P, Yamamoto S, Duh E, Zack DJ, Li Q, Berns KI, Raisler BJ, Hauswirth WW, Campochiaro PA: **AAV-mediated gene transfer of pigment epithelium-derived factor inhibits choroidal neovascularization**. *Invest Ophthalmol Vis Sci* 2002, **43**:1994–2000.

258. Liang H, Hou H, Yi W, Yang G, Gu C, Lau WB, Gao E, Ma X, Lu Z, Wei X, Pei J, Yi D: **Increased expression of pigment epithelium-derived factor in aged mesenchymal stem cells impairs their therapeutic efficacy for attenuating myocardial infarction injury**. *Eur Heart J* 2013, **34**:1681–1690.

259. Gao Y, Yao A, Zhang W, Lu S, Yu Y, Deng L, Yin A, Xia Y, Sun B, Wang X: **Human mesenchymal stem cells overexpressing pigment epithelium-derived factor inhibit hepatocellular carcinoma in nude mice**. *Oncogene* 2010, **29**:2784–2794.

260. Zolochevska O, Yu G, Gimble JM, Figueiredo ML: **Pigment Epithelial-Derived Factor and Melanoma Differentiation Associated Gene-7 Cytokine Gene Therapies Delivered by Adipose-Derived Stromal/Mesenchymal Stem Cells are Effective in Reducing Prostate Cancer Cell Growth**. *Stem Cells Dev* 2012, **21**:1112–1123.

261. Chen Q, Cheng P, Yin T, He H, Yang L, Wei Y, Chen X: **Therapeutic potential of bone marrow-derived mesenchymal stem cells producing pigment epithelium-derived factor in lung carcinoma**. *Int J Mol Med* 2012, **30**:527–534.

262. Chiellini C, Cochet O, Negroni L, Samson M, Poggi M, Ailhaud G, Alessi M-C, Dani C, Amri E-Z: **Characterization of human mesenchymal stem cell secretome at early steps of adipocyte and osteoblast differentiation**. *BMC Mol Biol* 2008, **9**:26.

263. Rodger J, Drummond ES, Hellström M, Robertson D, Harvey AR: **Long-term gene therapy causes transgene-specific changes in the morphology of regenerating retinal ganglion cells**. *PloS One* 2012, **7**:e31061.

264. Vargas AE, Markoski MM, Cañedo AD, da Silva FH, Nardi NB: **Genetic modification of mesenchymal stem cells**. *Methods Mol Biol Clifton NJ* 2012, **879**:479–490.

265. Gattu AK, Swenson ES, Iwakiri Y, Samuel VT, Troiano N, Berry R, Church CD, Rodeheffer MS, Carpenter TO, Chung C: **Determination of mesenchymal stem cell fate by pigment epithelium-derived factor (PEDF) results in increased adiposity and reduced bone mineral content**. *FASEB J* 2013, **27**:4384–4394.

266. Li F, Song N, Tombran-Tink J, Niyibizi C: **Pigment epithelium-derived factor enhances differentiation and mineral deposition of human mesenchymal stem cells**. *Stem Cells Dayt Ohio* 2013, **31**:2714–2723.

267. Becerra SP, Notario V: **The effects of PEDF on cancer biology: mechanisms of action and therapeutic potential**. *Nat Rev Cancer* 2013, **13**:258–271.

268. Wang Q, Zhang Z, Ding T, Chen Z, Zhang T: **Mesenchymal stem cells overexpressing PEDF decrease the angiogenesis of gliomas**. *Biosci Rep* 2013, **33**.

269. Chen Q, Cheng P, Song N, Yin T, He H, Yang L, Chen X, Wei Y: **Antitumor activity of placenta-derived mesenchymal stem cells producing pigment epithelium-derived factor in a mouse melanoma model**. *Oncol Lett* 2012, **4**:413–418.

270. Chen, Ye, Zhang, Jiang: **The molecular impact of pigment epithelium-derived factor, PEDF, on lung cancer cells and the clinical significance**. *Int J Oncol* 2009, **35**.

271. HE S-S, SHI H-S, YIN T, LI Y-X, LUO S-T, WU Q-J, LU L, WEI Y-Q, YANG L: **AAV-mediated gene transfer of human pigment epithelium-derived factor inhibits Lewis lung carcinoma growth in mice**. *Oncol Rep* 2012, **27**:1142–1148.

272. He S-S, Wu Q-J, Gong C, Luo S-T, Zhang S, Li M, Lu L, Wei Y-Q, Yang L: **Enhanced efficacy of combination therapy with adeno□associated virus-delivered pigment epithelium-derived factor and cisplatin in a mouse model of Lewis lung carcinoma**. *Mol Med Rep* 2014.

273. Li L, Yao Y-C, Fang S-H, Ma C-Q, Cen Y, Xu Z-M, Dai Z-Y, Li C, Li S, Zhang T, Hong H-H, Qi W-W, Zhou T, Li C-Y, Yang X, Gao G-Q: **Pigment Epithelial-derived Factor (PEDF)-triggered Lung Cancer Cell Apoptosis Relies on p53 Protein-driven Fas Ligand (Fas-L) Up-regulation and Fas Protein Cell Surface Translocation**. *J Biol Chem* 2014, **289**:30785–30799.

274. Mahtabifard A, Merritt RE, Yamada RE, Crystal RG, Korst RJ: **In vivo gene transfer of pigment epithelium-derived factor inhibits tumor growth in syngeneic murine models of thoracic malignancies**. *J Thorac Cardiovasc Surg* 2003, **126**:28–38.

275. Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG: **Down-regulation of PEDF expression by ribozyme transgene in endothelial and lung cancer cells and its impact on angiogenesis in vitro**. *Oncol Rep* 2005, **14**:1615–1619.

276. Feng C-C, Guan M, Wang P-H, Ding Q, Zhang Y-F, Jiang H-W, Wen H, Wu Z: **Pigment epithelium-derived factor inhibits T24 bladder cancer cell growth in vitro and in vivo**. *Romanian Biotechnol Lett* 2011, **16**.

277. Cai J, Parr C, Watkins G, Jiang WG, Boulton M: **Decreased Pigment Epithelium–Derived Factor Expression in Human Breast Cancer Progression**. *Clin Cancer Res* 2006, **12**:3510–3517.

278. Jan R, Huang M, Lewis-Wambi J: **Loss of pigment epithelium-derived factor: A novel mechanism for the development of endocrine resistance in breast cancer**. *Breast Cancer Res* 2012, **14**:R146.

279. Koskimaki JE, Rosca EV, Rivera CG, Lee E, Chen W, Pandey NB, Popel AS: **Serpin-Derived Peptides Are Antiangiogenic and Suppress Breast Tumor Xenograft Growth**. *Transl Oncol* 2012, **5**:92–97.

280. Hosomichi J, Yasui N, Koide T, Soma K, Morita I: **Involvement of the collagen I-binding motif in the anti-angiogenic activity of pigment epithelium-derived factor**. *Biochem Biophys Res Commun* 2005, **335**:756–761.

281. Yang J, Chen S, Huang X, Han J, Wang Q, Shi D, Cheng R, Gao G, Yang X: **Growth suppression of cervical carcinoma by pigment epithelium-derived factor via anti-angiogenesis**. *Cancer Biol Ther* 2010, **9**:967–974.

282. Jia L, Waxman DJ: **Thrombospondin-1 and pigment epithelium-derived factor enhance responsiveness of KM12 colon tumor to metronomic cyclophosphamide but have disparate effects on tumor metastasis**. *Cancer Lett* 2013, **330**:241–249.

283. Wu QJ, Gong CY, Luo ST, Zhang DM, Zhang S, Shi HS, Lu L, Yan HX, He SS, Li DD, others: **AAV-mediated human PEDF inhibits tumor growth and metastasis in murine colorectal peritoneal carcinomatosis model**. *BMC Cancer* 2012, **12**:129.

284. Li L, Yang J, Wang W-W, Yao Y-C, Fang S-H, Dai Z-Y, Hong H-H, Yang X, Shuai X-T, Gao G-Q: **Pigment epithelium-derived factor gene loaded in cRGD–PEG–PEI suppresses colorectal cancer growth by targeting endothelial cells**. *Int J Pharm* 2012, **438**:1–10.

285. Guan M, Yam HF, Su B, Chan KP, Pang CP, Liu WW, Zhang WZ, Lu Y: **Loss of pigment epithelium derived factor expression in glioma progression**. *J Clin Pathol* 2003, **56**:277–282.

286. Guan M, Pang C-P, Yam H-F, Cheung K-F, Liu W-W, Lu Y: **Inhibition of glioma invasion by overexpression of pigment epithelium-derived factor**. *Cancer Gene Ther* 2004, **11**:325–332.

287. Zhang T, Guan M, Xu C, Chen Y, Lu Y: **Pigment epithelium-derived factor inhibits glioma cell growth in vitro and in vivo**. *Life Sci* 2007, **81**:1256–1263.

288. Browne M, Stellmach V, Cornwell M, Chung C, Doll JA, Lee E-J, Jameson JL, Reynolds M, Superina RA, Abramson LP, Crawford SE: **Gene Transfer of Pigment Epithelium-Derived Factor Suppresses Tumor Growth and Angiogenesis in a Hepatoblastoma Xenograft Model**. *Pediatr Res* 2006, **60**:282–287.

289. Merritt RE, Yamada RE, Wasif N, Crystal RG, Korst RJ: **Effect of inhibition of multiple steps of angiogenesis in syngeneic murine pleural mesothelioma**. *Ann Thorac Surg* 2004, **78**:1042–1051.

290. Garcia M, Fernandez-Garcia N, Rivas V, Carretero M, Escamez MJ, Gonzalez-Martin A, Medrano EE, Volpert O, Jorcano JL, Jimenez B, Larcher F, Del Rio M.: **Inhibition of Xenografted Human Melanoma Growth and Prevention of Metastasis Development by Dual Antiangiogenic/Antitumor Activities of Pigment Epithelium-Derived Factor**. *Cancer Res* 2004, **64**:5632–5642.

291. Shi H, Yang L, Wei W, Su X, Li X, Li M, Luo S, Zhang H, Lu L, Mao Y, Kan B, Yang L: **Systemically administered liposome-encapsulated Ad-PEDF potentiates the anti-cancer effects in mouse lung metastasis melanoma**. *J Transl Med* 2013, **11**:86.

292. Yang L-P, Cheng P, Peng X-C, Shi H-S, He W-H, Cui F-Y, Luo S-T, Wei Y-Q, Yang L: **Anti-tumor effect of adenovirus-mediated gene transfer of pigment epithelium-derived factor on mouse B16-F10 melanoma**. *J Exp Clin Cancer Res* 2009, **28**:75.

293. Streck CJ, Zhang Y, Zhou J, Ng C, Nathwani AC, Davidoff AM: **Adeno-associated virus vector-mediated delivery of pigment epithelium-derived factor restricts neuroblastoma angiogenesis and growth**. *J Pediatr Surg* 2005, **40**:236–243.

294. Cheung LWT, Au SCL, Cheung ANY, Ngan HYS, Tombran-Tink J, Auersperg N, Wong AST: **Pigment Epithelium-Derived Factor Is Estrogen Sensitive and Inhibits the Growth of Human Ovarian Cancer and Ovarian Surface Epithelial Cells**. *Endocrinology* 2006, **147**:4179–4191.

295. Hase R, Miyamoto M, Uehara H, Kadoya M, Ebihara Y, Murakami Y, Takahashi R: **Pigment Epithelium-Derived Factor Gene Therapy Inhibits Human Pancreatic Cancer in Mice**. *Clin Cancer Res* 2005, **11**:8737–8744.

296. Guan M, Jiang H, Xu C, Xu R, Chen Z, Lu Y: **Adenovirus-mediated PEDF expression inhibits prostate cancer cell growth and results in augmented expression of PAI-2**. *Cancer Biol Ther* 2007, **6**:419–425.

297. Halin S, Rudolfsson SH, Doll JA, Crawford SE, Wikström P, Bergh A: **Pigment epithelium-derived factor stimulates tumor macrophage recruitment and is downregulated by the prostate tumor microenvironment**. *Neoplasia N Y N* 2010, **12**:336–345.

298. Liu W, Wu Z, Guan M, Lu Y: **cDNA microarray analysis of pigment epithelium-derived factor-regulated gene expression profile in prostate carcinoma cells: Gene expression changes induced by PEDF**. *Int J Urol* 2009, **16**:323–328.

299. Nelius T, Samathanam C, Martinez-Marin D, Gaines N, Stevens J, Hickson J, de Riese W, Filleur S: **Positive correlation between PEDF expression levels and macrophage density in the human prostate**. *The Prostate* 2013, **73**:549–561.

300. Abramson LP, Browne M, Stellmach V, Doll J, Cornwell M, Reynolds M, Arensman RM, Crawford SE: **Pigment epithelium–derived factor targets endothelial and epithelial cells in Wilms' tumor**. *J Pediatr Surg* 2006, **41**:1351–1356.

301. Broadhead ML, Dass CR, Choong PFM: **Systemically administered PEDF against primary and secondary tumours in a clinically relevant osteosarcoma model**. *Br J Cancer* 2011, **105**:1503–1511.

302. Broadhead ML, Choong PFM, Dass CR: **Efficacy of Continuously Administered PEDF-Derived Synthetic Peptides against Osteosarcoma Growth and Metastasis**. *J Biomed Biotechnol* 2012, **2012**:1–10.

303. Dass CR, Contreras KG, Dunstan DE, Choong PFM: **Chitosan microparticles encapsulating PEDF plasmid demonstrate efficacy in an orthotopic metastatic model of osteosarcoma**. *Biomaterials* 2007, **28**:3026–3033.

304. Dass CR, Ek ET, Choong PF: **PEDF as an emerging therapeutic candidate for osteosarcoma**. *Curr Cancer Drug Targets* 2008, **8**:683–690.

305. Ek ET, Dass CR, Contreras KG, Choong PF: **Pigment epithelium-derived factor overexpression inhibits orthotopic osteosarcoma growth, angiogenesis and metastasis**. *Cancer Gene Ther* 2007, **14**:616–626.

306. Ta HT, Dass CR, Larson I, Choong PFM, Dunstan DE: **A chitosan hydrogel delivery system for osteosarcoma gene therapy with pigment epithelium-derived factor combined with chemotherapy**. *Biomaterials* 2009, **30**:4815–4823.