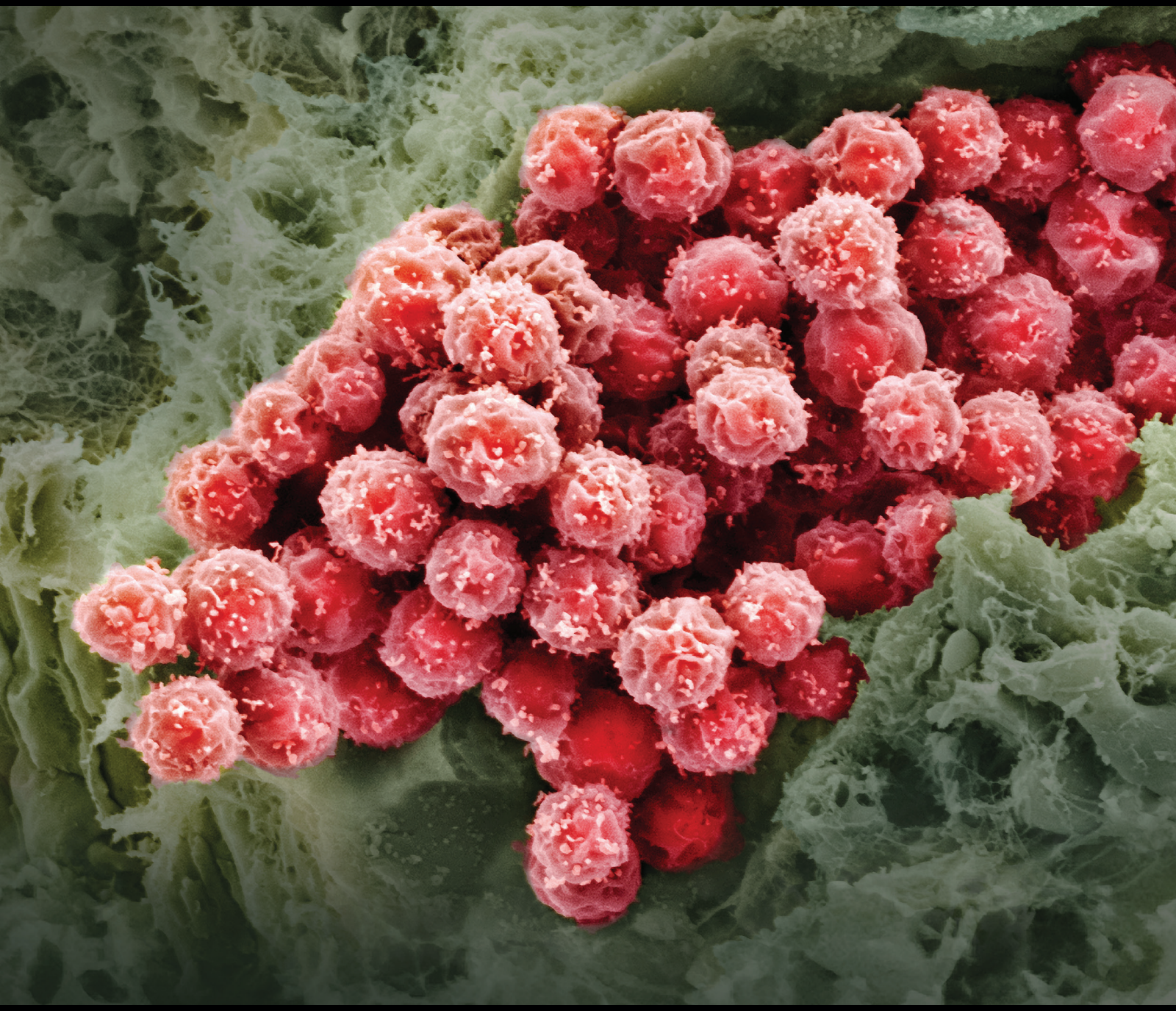
Stem Cells International

MSCs: Scientific Support for Multiple Therapies

Guest Editors: Mark F. Pittenger, Katarina Le Blanc, Donald G. Phinney, and Jerry K. Y. Chan



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Editorial Board

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Editorial

MSCs: Scientific Support for Multiple Therapies

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Whether we call them Mesenchymal Stem Cells, Mesenchymal Stromal Cells, Multipotent Stromal Cells, Skeletal Stem Cells, or another name, this class of stem/progenitor cells has been continuously studied for more than 20 years. Over 30,000 papers have been published on MSCs and over 10,000 patients have been administered MSC or MSC-like cells in over 300 clinical trials. Importantly, the first autologous *in vitro* cultured MSCs were injected into recipients over two decades ago, and the safety record for MSCs remains strong. Nevertheless, there remains much to learn about MSC science and their therapeutic potential. For example, the varied approaches taken by different laboratories to exploit the MSC therapeutic potential speaks to the complexity of MSCs and the ingenuity of those that study the cells. One size does not fit all.

The ease of isolation and propagation of MSCs means that anyone can become proficient in culturing MSCs but there is a learning curve for MSC assay reproducibility. However, once this proficiency is achieved new results can be produced with confidence. The field continues to seek new assays and metrics that reliably and reproducibly predict the therapeutic efficacy of a MSC preparation. Also, a typical clinical dose of MSCs is 100 million cells in 30–50 mL, which is approximately 400 uL of packed cells. Therefore, a 70 kg patient is typically administered 0.5 mL of adult stem cells but once injected this dose of cells diminishes quickly such that within ~48 hrs only 10% may be left, further diminishing to 1-2% over the next 48 hrs. Therefore, it is quite remarkable that the administered MSCs have any therapeutic effect. Consequently, it is critical to further improve our measurement of the survival of transplanted cells and their biodistribution to better predict their clinical efficacy and potency.

In this issue, seven MSC research groups present new work or focused reviews on MSCs.

The acknowledged founder of the MSC field and one of its strongest advocates, A. I. Caplan, has thought carefully about MSCs for many decades and therefore his perspectives on the field at large are always informative. Bone repair played a major role in the discovery and initial studies of MSCs and bone tissue serves as a clear example of where MSCs participate and persist in the healed tissue. Here, A. I. Caplan reminds us that MSCs or MSC-like cells are found in all tissues and provide local control over inflammation and tissue repair by facilitating the actions of resident cells and modifying the milieu of factors at sites of repair and regeneration.

Over 300 MSC-based clinical trials have been registered at http://clinicaltrials.gov/. As N. Escacena et al. point out the large number of clinical studies reported have varied in their efficacy and outcomes, demonstrating that we still have ways to go to use MSCs effectively across many therapeutic areas. Knowing more about the patients to be treated as well as the tissue source and method of MSC preparation and their therapeutic administration should improve the overall results of clinical studies. While this is clearly desirable, the early death of administered MSCs and its effects on

the patient's physiology need careful consideration. At this juncture, MSCs are used primarily for their cytokine and growth factor production rather than for their cell replacement and differentiation ability. There are several reasons for this approach including the following: (1) MSCs secrete powerful anti-inflammatory factors and all tissue injury is accompanied by inflammation; (2) MSCs produce angiogenic factors and interaction with other cell types further enhances their production of these factors, which are needed during tissue repair; (3) MSCs become adapted to *in vitro* metabolic conditions; and (4) most MSCs do not survive the quick transition to *in vivo* conditions. Therefore, the field still needs reliable measures of cell survival and engraftment both short term and long term following administration to patients.

S.-Y. Kao et al. isolated MSCs from pancreatic tissue as well as bone marrow and Wharton's jelly and studied their differentiation into insulin producing cells. These authors have previously generated insulin producing cells to treat diabetic mice and rats and this study tested the effects of insulin producing MSCs from different sources in the streptozotocin-induced diabetic rats.

M. Ferretti et al. provide a study of the interaction of chondrocytes isolated from osteoarthritic patients with their bone marrow derived MSCs. The interaction of the two cell types provides much more production of hyaluronan, an important player in joint healing. This is a theme that persists in cell therapy but remains underappreciated, in which cell-cell interactions provide a dynamic environment not evident when single cell types are studied or applied to damaged tissue. As we develop expertise in single cell therapy, intentional combination cell therapies will need to be carefully tested.

Low level laser therapy has been available clinically for several decades and has been used to stimulate wound healing and hair growth. Mechanistically, laser light can increase collagen synthesis or stimulate IL-8 and IL-1α production. T. Kushibiki et al. provide a study of laser light effects on MSCs and osteogenic differentiation. Laser light provides another tool that many investigators are not familiar with to stimulate dynamic changes in MSCs.

For several years, W. C. W. Chen et al. have studied the multipotent cells available in the vasculature associated with the intima, media, and adventitia layers of vessels, collectively known as microvascular pericytes. The culture of these pericytes results in MSC-like cells when various assays are performed. The availability of these reparative cells along all vessels raises the possibility of immediately available *in situ* regenerative cells when injury occurs. Here the authors review the characterization and regenerative potential of pericytes. As tissue injury healing slows with age, the question remains why bone marrow MSCs and microvascular pericytes work so well for tissue healing when we are young, but not after we grow old.

The case has been made in the past that MSCs may promote tumor growth due to their trophic effects and production of angiogenic factors. MSCs have a propensity to migrate to wounds and damaged tissue, and tumors are characterized as wounds that do not heal. With this in mind, gene modified MSCs have been tested for their ability to deliver anticancer compounds in experimental models. Past work in this area has shown that MSCs isolated from patients with myeloproliferative disorders do not have the identified oncogenic JAK2 mutation suggesting that MSCs and the tumorigenic hematologic progenitor cells did not share a mutated common ancestor. As MSCs migrate to tumors, other researchers have used them to deliver interferon β to tumors. In this issue P. Johann and I. Müller review the evidence that MSCs may aid solid tumor growth and compare them with tumor associated fibroblasts that have a longer literature history than the more recently described MSC.

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Research Article

Comparisons of Differentiation Potential in Human Mesenchymal Stem Cells from Wharton's Jelly, Bone Marrow, and Pancreatic Tissues

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Background. Type 1 diabetes mellitus results from autoimmune destruction of β-cells. Insulin-producing cells (IPCs) differentiated from mesenchymal stem cells (MSCs) in human tissues decrease blood glucose levels and improve survival in diabetic rats. We compared the differential ability and the curative effect of IPCs from three types of human tissue to determine the ideal source of cell therapy for diabetes. *Methods*. We induced MSCs from Wharton's jelly (WJ), bone marrow (BM), and surgically resected pancreatic tissue to differentiate into IPCs. The *in vitro* differential function of these IPCs was compared by insulin-to-DNA ratios and C-peptide levels after glucose challenge. *In vivo* curative effects of IPCs transplanted into diabetic rats were monitored by weekly blood glucose measurement. *Results*. WJ-MSCs showed better proliferation and differentiation potential than pancreatic MSCs and BM-MSCs. *In vivo*, WJ-IPCs significantly reduced blood glucose levels at first week after transplantation and maintained significant decrease till week 8. BM-IPCs reduced blood glucose levels at first week but gradually increased since week 3. In resected pancreas-IPCs group, blood glucose levels were significantly reduced till two weeks after transplantation and gradually increased since week 4. *Conclusion*. WJ-MSCs are the most promising stem cell source for β-cell regeneration in diabetes treatment.

1. Introduction

Diabetes mellitus (DM) is one of the leading causes of death in the world and more and more people suffer from this widespread disease [1, 2]. It has been recognized that Type 1 DM, called juvenile-onset diabetes, develops due to beta cells being attacked and destroyed by the individual's own immune system. Type 2 DM, adult-onset diabetes, is characterized by insulin resistance, resulting in the ineffectiveness of insulin [3]. Both types of DM lead to rising blood glucose levels, which are associated with many complications such as retinopathy, nephropathy, and neuropathy, among others [4]. Subcutaneous injections of insulin are commonly used to manage Type 1 DM as well as the later stage of Type 2 DM. However, the requirements of frequent insulin injections and troublesome blood glucose monitoring have been criticized, as well as the lack of cure for diabetes [5].

Transplantation of cadaveric pancreases began to flourish in 1966, allowing people with diabetes to live without

insulin injections [6]. As the success rate of transplantation increased, it became widely popular. However, disadvantages were noted, including shortage of donor pancreases, a certain degree of surgical risk, and high risk of complications after patients received life-long immunosuppression. Therefore, pancreas islet transplantation emerged as another possible solution for diabetes. Currently, the most effective protocol is the Edmonton protocol, which involves a one-time injection of *in vivo* expanded islet cells from at least two donors via the portal vein. Though the procedure reduces surgical risks, its therapeutic effect can only be sustained for about 10 years with an insulin independence rate of not more than 15%. Also, the demand for donor islets still outweighs their availability. Meanwhile, long-term use of immunosuppressants is still necessary accompanied by its inevitable side effects [7].

In recent years, researchers have made great efforts to develop regeneration therapy, in which stem cells or endocrine precursor cells are stimulated to differentiate into insulin-producing cells (IPCs) for replacing destroyed β cells. Regeneration therapy progresses rapidly because it has potentially fewer limitations in comparison to the above two therapeutic strategies [8].

In general, the ideal tissue source for regeneration therapy for diabetes must meet certain criteria such as abundant availability, easy duplication, and equivalent function to that of the primary beta cell. Not only embryonic stem cells, but also adult stem cells, adult human pancreatic precursor cells, and extrapancreatic endocrine progenitor cells have been reported as surrogate β-cells in the literature [8, 9]. Although great advances have been achieved in generating β-cells, there is not yet any consensus on what kind of cell source best meets the requirements for treating diabetes.

Our laboratory has demonstrated that pancreatic endocrine precursor (PEP) cells can be generated from Wharton's jelly mesenchymal stem cells (WJ-MSCs) [10, 11], bone marrow mesenchymal stem cells (BM-MSCs) [12], and surgically resected adult pancreatic tissues [13]. PEP cells can be induced into IPCs and are able to reverse hyperglycemia after transplantation in STZ-induced diabetic rats. The aim of this study was to compare the *in vitro* differential ability and the *in vivo* curative effect of IPCs generated from different sources, including Wharton's jelly, BM, and pancreatic tissues, to determine the ideal source of cell therapy for treatment of diabetes.

2. Methods

2.1. Isolation and Differentiation of IPCs from Resected Human Pancreatic Tissue. Institutional Review Board approval (Taipei Veterans General Hospital) was obtained for all procedures. With the written informed consent of the parents, the healthy pancreatic parenchyma tissue was resected from the normal portion which was used for anastomosis. To prevent degradation, the fresh pancreatic tissue was initially preserved in solution D (0.137 M NaCl, 5.38 mM KCl, 0.19 mM Na_2HPO_4, 0.205 mM K_2HPO_4, 5.49 mM glucose, 0.058 M sucrose, 1% penicillin/streptomycin, and 0.12% fungizone). The tissue was then minced and digested by 2 mg/mL Type V collagenase (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. The digested sample was washed three times with cold Dulbecco's modified Eagle medium/F12 (DMEM/F12, Invitrogen, Carlsbad, CA). After centrifugation at 1200 g for 20 minutes at 4°C in Histopaque (1.077 mg/mL) and DMEM/F12 gradients, pancreatic duct cells, islets, and endocrine precursor cells (EPCs) were isolated. The EPCs from the Histopaque/DMEM interface were aspirated and washed with DMEM/F12 and then cultured with CMRL 1066 medium (5.5 mM glucose, Invitrogen corporation) containing 10% FBS, 1% penicillin/streptomycin, 100 ng/mL nerve growth factor (R&D Systems, Minneapolis, MN), 10 mM nicotinamide (Sigma), and 25 ng/mL epidermal growth factor (EGF, Invitrogen). After 7–10 expansion days, the EPCs reached confluence. The EPCs were trypsinized with 0.05% trypsin/EDTA (Invitrogen), washed with serum-free DMEM/F12 (17.5 mmol/l glucose), and seeded into 6-well culture dishes coated with Matrigel (BD Bioscience, Bedford, MA, USA) for further culture and differentiation. The number of the EPCSs in each well was 1×10^6 cells. Insulin, transferrin, sodium selenite + linoleic acid (ITS + l, Sigma), 2 g/L BSA, and 10 ng/mL basic fibroblastic growth factor (bFGF, Invitrogen) were added in the culture medium. After 5–7 days in Matrigel, the cells aggregated from monolayers to clusters and differentiated into IPCs. The gel layer was then disrupted with a cell scraper. Both the IPC clusters and the Matrigel pieces were transferred to a large volume of prewarmed medium and individual cell clusters were handpicked with a fire-polished glass pipette. The IPC clusters were then kept in suspension 5 days in serum-free DMEM/F12 supplemented with ITS + l [13].

2.2. Isolation and Differentiation of IPCs from BM-MSCs. All study procedures were approved by the Institutional Review Board (Taipei Veterans General Hospital). Bone marrow tissues were gathered from 20 healthy donors with their informed consent. After washing the bone marrow sample twice with phosphate buffered saline (PBS, PH = 7.2), density gradient centrifugation (NycoPrep 1.077, Axis-Shield, Oslo, Norway) was possessed and BM-MSCs were isolated. Rinse the BM-MSCs twice in low glucose DMEM (LG-DMEM, 5.5 mM glucose, Invitrogen, Carlsbad, CA) and culture them at 37°C with 5% humidified CO2 in expansion medium consisting of L-DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin/Amphotericin (Biological Industries, Haifa, Israel). The culture medium was replaced every 3 days and the nonadherent cells were removed. When the adherent BM-MSCs were 90–95% confluent (10–15 days), they were subcultured by Trypsin-Versene (Invitrogen). When the third passage BM-MSC reached 80% confluence, it was provided to differentiate into IPCs by culturing in serum-free high glucose DMEM (HG-DMEM, 25 mM glucose) supplemented with 1% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). 3 days after, the culture medium was replaced with HG-DMEM supplemented with 10% FBS for another 14 days [12].

2.3. Isolation and Differentiation of IPCs from WJ-MSCs. All study procedures were approved by the Institutional Review

Board (Taipei Veterans General Hospital). With the written informed consent of the parents, fresh human umbilical cords were obtained after birth and stored in Hank's balanced salt solution (Biological Industries, Israel) prior to tissue processing to obtain MSCs. After removal of blood vessels, the mesenchymal tissue was scraped off the Wharton's jelly and centrifuged at 250 g for 5 min. After centrifugation, the pellets were resuspended in 15 mL of serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 0.2 g/mL of collagenase and incubated for 16 h at 37°C. Next, the cells were washed, resuspended in DMEM containing 2.5% trypsin, and incubated for 30 min at 37°C with agitation. Finally, cells were again washed and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma St. Louis, MO, USA) and glucose (4.5 g/L) in 5% CO_2 in a 37°C incubator. At the fourth to sixth passage, after reaching a confluence of 70%, the MSCs were induced to differentiate into islet-like cell aggregates with three stages. Undifferentiated MSCs were detached by HyQTase, diluted with SFM-A, and centrifuged. Cells were counted for initial seeding density and 1×10^6 cells/cm^2 were resuspended in SFM-A and seeded on ultralow attachment tissue culture plates (Corning, Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com/). SFM-A contained DMEM/F12 (1 : 1) (Gibco, Grand Island, NY) with 17.5 Mm glucose, 1% BSA Cohn fraction V, fatty acid free (Sigma-Aldrich), 1% penicillin/streptomycin/amphoteric B (PSA; Biological Industries, Israel), insulin-transferrin-selenium-X (ITS-X; 5 mg/L insulin, 5 mg/L transferrin, 5 mg/L selenium), 4 nM activin A, 1 mM sodium butyrate, and 50 μM 2-mercaptoethanol. The cells were cultured in this medium for 2 days. On the third day, the culture medium was changed to SFM-B, which contains DMEM/F12 (1 : 1) with 17.5 mM glucose, 1% BSA, 1% PSA, ITS-X, and 0.3 mM taurine. On the fifth day, the cell culture was replaced by SFM-C, which contained DMEM/F12 (1 : 1) with 17.5 mM glucose, 1.5% BSA, ITS-X, 1% PSA, 3 mM taurine, 100 nM glucagon-like peptide (GLP)-1 (amide fragment 7–36; Sigma Aldrich), 1 mM nicotinamide, and nonessential amino acids (NEAAs). For the next 5 days, the culture medium was exchanged with fresh SFM-C every 2 days [11].

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-Time PCR Analysis to Determine Gene Expressions in Differentiated Cells. To determine whether the three kinds of cell sources had differentiated into IPCs, the expressions of genes involved with both pancreatic β-cell development and insulin production were examined by RT-PCR and real-time PCR. All RNA extractions from the three types of differentiated cells were performed with Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 4 mg RNA using the Superscript TM III first-strand synthesis system (Invitrogen). PCR was performed with 200 ng RNA equivalents using specific primers in the presence of SYBR Green I (LightCycler TM-FastStart DNA Master SYBR Green I; Roche, Basel, Switzerland). Primers were Pdx1 forward GGAGCCGGAGGAGAACAAG, reverse CTCGGTCAAGTTCAACATGACAG; Pax4 forward GGGTCTGGTTTTCCAACAGAAG, reverse CAGCGCTGCTGGACTT; Glut2 forward GCCTAGTTATGCATGCAG, reverse GGTTTGTAACTTATGCCTAAG; insulin forward ACCAGCATCTGCTCCCTCTA, reverse GGTTCAAGGGCTTTATTCCA; GAPDH forward CACCATCTTCCAGGAGCGAG, reverse TCACGCCACAGTTTCCCGGA (Mission Biotech, Taiwan). A LightCycler 480 (Roche, Indianapolis, IN) was used for real-time PCR with the following cycling program: 50°C for 2 min, 95°C for 10 min, and 35 cycles at 95°C for 15 s and 60°C for 1 min. Melting curves were obtained at 60°C. The number of PCR cycles was titrated in order to remain in the linear range of amplification. The resultant amplification products (10 mL) were separated using 2% agarose gel electrophoresis and were visualized with ethidium bromide that validate the specificity of the real-time PCRs [11–13].

2.5. Measurement of Insulin-to-DNA Ratio. The three types of differentiated cells were washed twice with PBS, resuspended in 300 mL of distilled cold water, and homogenized by sonication on ice. An aliquot of the homogenates was analyzed fluorometrically for DNA content in duplicate, and another aliquot was extracted with acid ethanol overnight and measured for insulin content using an ELISA kit (Mercodia, Uppsala, Sweden).

2.6. Measurement of C-Peptide Level after Glucose Challenge Test. The three types of differentiated cells were incubated for 1 h in DMEM-LG (5.5 mM glucose) and the medium was collected and stored at −20°C. The cells were washed with PBS and incubated for 1 h in DMEM-HG (25 mM glucose) (Gibco, NY) and the medium was collected and stored at −20°C. The C-peptide concentration was determined by C-peptide ELISA kit (Mercodia, Uppsala, Sweden).

2.7. Comparison of In Vivo Curative Effect by Intrahepatic IPCs Injection in STZ-Induced Diabetic Rats. Hyperglycemia was induced in 24 male SD rats of closed colony (body weight 300–350 g) through intraperitoneal injection of 30 mg/kg of streptozotocin (STZ) on 3 consecutive days. Blood glucose levels were determined using Roche ACCU-CHEK glucose meter (Roche Diagnostics, Indianapolis, IN, USA.) by tapped tail-vein blood. Stable hyperglycemia (blood glucose levels ranging between 16.7 and 33.3 mmol/L) developed in 24 rats one week later. The 18 diabetic rats, 6 in each of the three study groups, were anesthetized with pentobarbital (40 mg/kg, i.p.). After midline laparotomy, the portal vein was identified and 0.5 mL heparinized saline and 5×10^6 differentiated insulin-producing cells (three types of differentiated cells) suspended in 0.1 mL of normal saline were injected into the catheters of WJ-MSCs, BM-MSCs, and pancreatic MSCs study groups, followed by a volume of normal saline equivalent to the volume of the Port-A-Cath catheter (0.35 mL) to push the grafts into the portal vein. The 6 rats in the STZ group underwent the same procedure but were only injected with normal saline (STZ group). Body weight and blood sugar levels

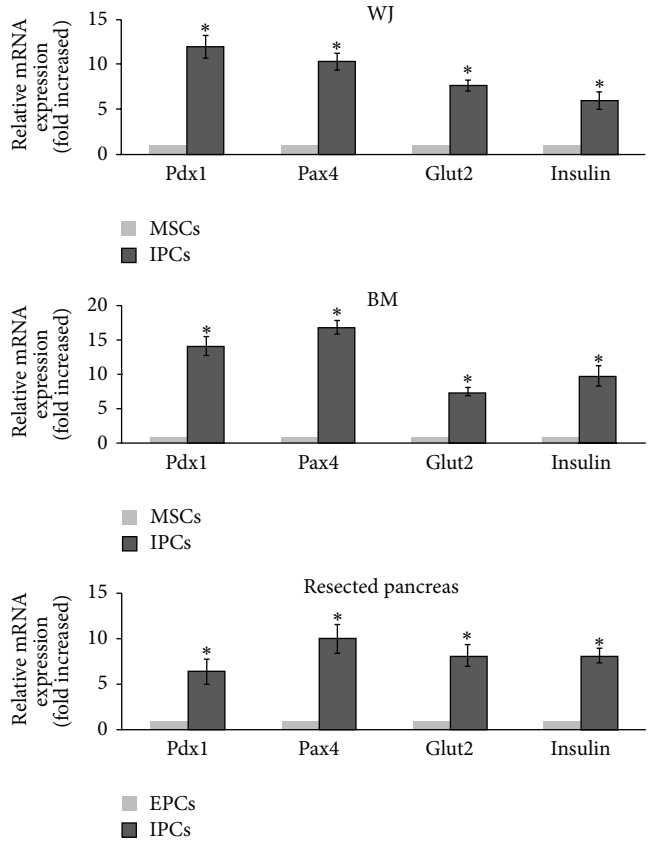

Figure 1: Real-time PCR analyses of three kinds of cell sources after differentiation to evaluate the expression of pancreatic β-cell development-related and insulin production-related genes, including Pdx1, Pax4, Glut2, and Insulin. Results are the means ± SD for 6 experiments. *: $P < 0.05$ compared to nondifferentiated cells.

were recorded before and after cell transplantation. Blood was collected from a tail vein and blood glucose levels were measured with a blood glucose meter (Roche, Basel, Switzerland) [11–13].

2.8. Immunofluorescence Analysis. The rats were sacrificed 8 weeks after transplantation and perfused with 4% formaldehyde (Ferak, Berlin, Germany). The pancreatic tissues were resected and cut into 0.5–1.0 cm^3 pieces. The samples were dehydrated and embedded in OCT (Sakura Finetek USA Inc., Torrance, CA, USA) in liquid nitrogen. The cryosections (5 μm/piece) were washed twice with PBS and incubated overnight at 4°C with rabbit anti-human C-peptide antibodies (1 : 100; Santa Cruz, Santa Cruz, CA, USA). After 3 washes in PBS, slides were incubated for 1 h at room temperature with Cy3-labeled goat anti-rabbit IgG (1 : 200, Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were counterstained using DAPI (1 : 5000, Molecular Probes, Inc., Eugene, OR, USA). After the sections were mounted with mounting medium (Vector Laboratories, Burlingame, CA, USA), microscopy was performed using a confocal microscope equipped with difference interference contrast light path (LSM 510, Zeiss, Göttingen, Germany).

2.9. Statistical Analysis. Each series of experiments was performed in triplicate. The results obtained from a typical experiment were expressed as the means ± standard deviation (SD). Statistical analysis was carried out using the SPSS 14.0 software program (Statistics Package for Social Sciences, SPSS Inc. Chicago, IL, USA). Statistical analysis used nonparametric Mann-Whitney U test (2 independent samples). A P value of less than, or equal to, 0.05 was established as statistical significance.

3. Results

3.1. Isolation, Cultivation, and Differentiation of Resected Human Pancreatic Tissue, Human BM-MSCs, and WJ-MSCs (See Figure 5). To determine whether the three kinds of cell sources had differentiated into IPCs, the expression of genes involved in pancreatic β-cell development and insulin production was examined by reverse transcriptase-PCR and real-time PCR. As shown in Figure 1, the pancreatic β-cell development related genes, including Pdx1, Pax4, Glut2, and insulin, were significantly expressed in differentiated IPCs greater than in undifferentiated cells from the three kinds of cell sources. It was a guarantee that they were real IPCs after our differentiation procedures. Besides, as described in the previously published articles, surgical pancreatic MSCs stained positive for C-peptide after 23 days of culturing in differentiation medium with an overall culture success rate

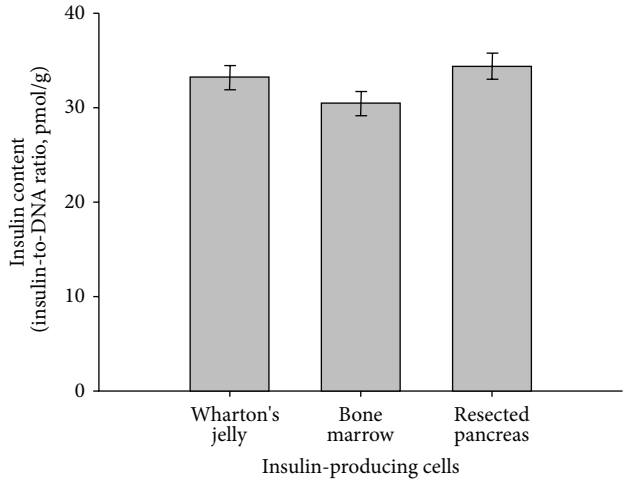

Figure 2: Insulin-to-DNA ratio in three different types of human tissue MSCs. Insulin-producing cells cultured in the maturation phase were stimulated as indicated and C-peptide in the medium was analyzed using ELISA. Measurements were normalized for DNA content of each sample. Three samples per condition were measured. Similar results were obtained in at least three independent experiments.

of 35% and differentiation potential of only one passage [13]. BM-MSCs stained positive for C-peptide at days 14 and 18 of differentiation, with greater expression on day 14 and differentiation potential of 4–6 passages [12]. WJ-MSCs stained with anti-human C-peptide antibodies showed that C-peptide was expressed at days 5 and 10 of differentiation, with greater expression on day 10 and differentiation potential of 8–10 passages [11].

3.2. Detection of Insulin/DNA Ratio in Differentiated Cells Derived from Resected Human Pancreatic Tissue, Human BM-MSCs, and WJ-MSCs. The expression of insulin/DNA ratio was greatest in human resected pancreas-IPCs compared to human WJ -IPCs and BM-IPCs. The expression of insulin/DNA ratio was greater in WJ-IPCs than in BM-IPCs. However, no significant differences were found in above comparisons (Figure 2).

3.3. Comparison of C-Peptide Secretion by Differentiated Cells in Response to Glucose Stimulation. To test whether the three kinds of IPCs have functional characteristics of pancreatic beta cells, we determined the secretion of C-peptide by each kind of IPCs at both low glucose concentration (5.5 mM) and high glucose concentration (25 mM). At a low glucose concentration, the IPCs from WJ-MCSs showed the highest release of C-peptide compared to IPCs from BM-MCSs and resected pancreas EPCs. At a high glucose concentration, the IPCs from WJ-MCSs showed the similar release of C-peptide as IPCs from BM-MCSs, both of which were higher than IPCs from resected pancreas EPCs. On the other hand, all of the three kinds of IPCs could significantly increase the C-peptide secretion in response to higher glucose stimulation. IPCs from the resected pancreas EPCs showed at least 10-fold increases in stimulated C-peptide secretion in response to high glucose. The IPCs from BM-MSCs revealed at least 4-fold increases in stimulated C-peptide secretion in response to high glucose. The IPCs from WJ-MSCs produced approximately twice the amount of C-peptide secretion at a high glucose concentration (Figure 3).

3.4. Changes in Blood Glucose in Rats after Transplantation of IPCs Derived from Resected Pancreas, BM-MSCs, and WJ-MSCs. In our experiments, the fasting blood glucose levels in STZ-induced diabetic rats were >350 mg/dL for one week and then increased to >400 mg/dL. The fasting blood glucose levels after STZ induction in the 4 groups were not the same, though they were not significantly different. In order to further normalize the post-STZ blood glucose level, we reported the change of fasting blood glucose levels between the specific time point and the day after STZ induction instead of the exact fasting blood glucose levels at the specific time point. The trends of blood glucose level of the three study groups after IPCs transplantation were all significantly different from the control group which was consistent with our previous study results. In the WJ-IPCs group, blood glucose levels were significantly reduced since the first week after transplantation, reached the maximal decrease at week 2, and maintained significant decrease till the end point of this study, 8 weeks after transplantation. In the BM-IPCs group, blood glucose levels were also significantly reduced since the first week after transplantation, reached the maximal decrease at week 2, but gradually increased since week 3. In the resected pancreas-IPCs group, blood glucose levels were significantly reduced till two weeks after transplantation, reached the maximal decrease at week 3, and gradually increased since week 4 (Figure 4).

3.5. Immunofluorescence Analysis of Pancreas in Rats after Transplantation of IPCs Derived from Resected Human Pancreatic Tissue, BM-MSCs, and WJ-MSCs. Groups of cells expressing green fluorescent protein (GFP) and human C-peptide were detected in the pancreas of STZ rats 8 weeks after transplantation. We successfully found that the WJ-IPCs had homing capacity to the pancreas. However, we could not

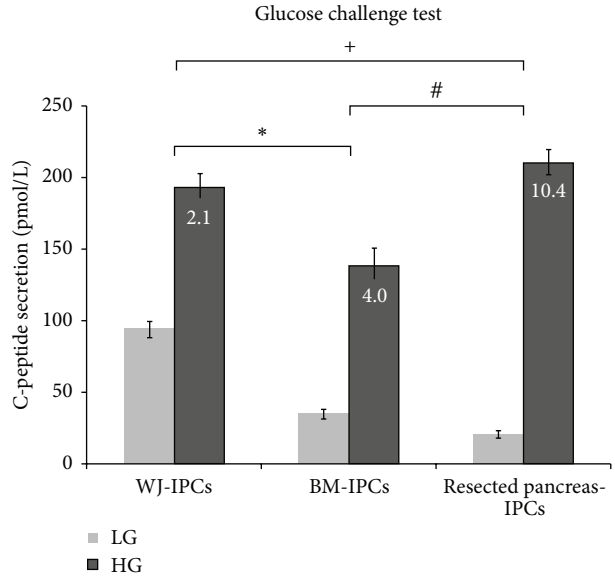

Figure 3: Secretion of C-peptide by cultured human IPCs in response to glucose stimulation. Glucose challenge test for C-peptide release in response to low glucose (LG: 5.5 mM) and high glucose (HG: 25 mM) concentrations of differentiated cells. ($^{+}$: the increasing fold of resected pancreas compared to the increasing fold of Wharton's jelly, $P < 0.05$; $^{\#}$: the increasing fold of resected pancreas compared to the increasing fold of bone marrow, $P < 0.05$; *: the increasing fold of bone marrow compared to the increasing fold of Wharton's jelly).

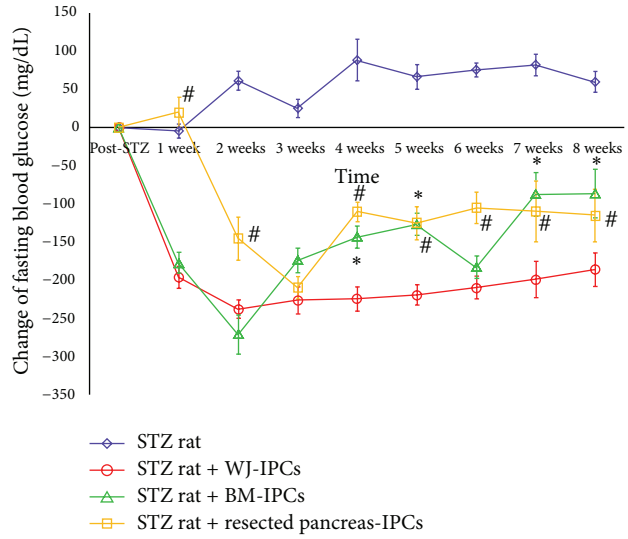

Figure 4: Changes in blood glucose levels in STZ-induced diabetic rats (study group: transplantation of IPCs into the portal vein via the Port-A-Cath with Wharton's jelly MSCs, BM-MSCs, and resected pancreas; STZ group: STZ-induced diabetic rats without transplantation of insulin-producing cells) Results are presented as the mean ± SD for 6 rats. *: $P < 0.05$ BM-IPCs group compared to WJ-IPCs group; $^{\#}$: $P < 0.05$ resected pancreas-IPCs compared to WJ-IPCs group.

find any IPCs in the pancreas of STZ rats which received transplantation with BM-IPCs and resected pancreas-IPCs.

4. Discussion

Since the shortage of organ donors has hampered the progress of pancreas transplantation as well as islet transplantation, alternative sources of insulin-producing cells are mandatory to overcome this hurdle. Stem cell regeneration has become a potential insulin replacement therapy. Stem cells from the pancreas [13–16], bone marrow [12, 17], umbilical cord blood [18], and embryo [19] have been used in research on regeneration therapies for DM.

Islet-like endocrine precursor cells (EPCs), one of the cell sources for regeneration therapy, are believed to exist either in pancreatic duct cells or in the islets themselves [20, 21]. The pancreas is composed of endocrine and exocrine compartments [22]. The endocrine compartment consists of

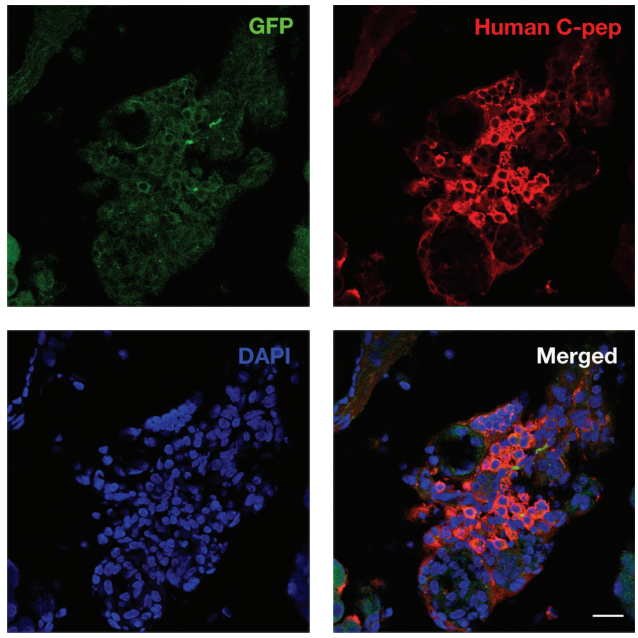

Figure 5: WJ-MSCs differentiated into IPCs and resided in the pancreas of the STZ rat. Confocal analysis of the pancreas tissues 23 days after WJ-IPCs transplantation showed clusters of cells with GFP expression and human C-peptide expression (red) as well as the nuclear stain, DAPI (blue). Scale bar = 50 μm.

α, β, δ, and pancreatic polypeptide cells, which are made up of islets, whereas the exocrine compartment contains acinar and ductal cells. Several studies have disclosed an interesting insight into the potential capability of the ductal cells to generate islet-like endocrine precursor cells, which develop into islet cells [23–28]. The islet-like endocrine precursor cell is thought to be one of the sources of new islet cells in adulthood [29].

MSCs, another type of promising stem cells, were first isolated from BM [30] and were found to have the potential to differentiate into different cell lineages, not only pancreatic β cells [10, 31], but also muscle cells, adipocytes, osteocytes, chondrocytes [32, 33], and cardiomyocytes [23–26]. They can be incorporated into a variety of tissues, including bone [27, 28], muscle [34], lung [34], and epithelium [35] following systemic injection. Studies have shown that IPCs can be developed from BM-MSCs [36], adipose tissue-derived MSCs [37], and human umbilical cord blood-derived mononuclear cells [18], which suggest their potential for use in autotransplantation. Recently, researchers have focused on isolating and cultivating the islet-like endocrine precursor cells in both fetal and adult pancreatic tissues [38, 39].

McElreavey et al. [40] reported the isolation of fibroblast-like cells from Wharton's jelly of the human umbilical cord, which are similar to BM-MSCs [41]. Researchers endeavored to obtain, characterize, and evaluate WJ-MSCs both *in vitro* and *in vivo*. The WJ-MSCs held many unique features of mesenchymal stromal cells such as the well-known surface phenotype, the plastic adherence, and the multipotency [42–45]. In fact, in appropriate *in vitro* stimulation, they could differentiate into adipocytes, osteoblasts, chondrocytes, hepatocytes, and cardiac and neural cells. They were considered to be ready for a new source for therapeutic materials [10].

In previous studies of our research team, insulin-producing cells were generated from Wharton's jelly, bone marrow, and human pancreatic tissues. Our cultivation and differentiation protocols referred to both predecessors in the literature and editors of the journals to which we submitted our reports. Owing to the distinct cell sources, different cultivation and differentiation protocols were applied. Nonetheless, we believed that the insulin-producing cells we generated from the three different sources were in their optimal condition after our specific differentiation methods. Next, we tried to investigate the cells' *in vitro* ability to secrete insulin and the *in vivo* curative effects of the transplanted cells in diabetic rats.

Human pancreatic stem cells *in vitro* seem to have the highest insulin/DNA ratio and are most affected by high glucose stimulation. However, these cells seemed to have a low success rate in the culture medium and difficulty in growing. In the present study, the successful culture rate of surgically resected pancreatic tissue was 35% and the differentiation potential was only one passage. Unsurprisingly, similar reports are found in the literature [46–48]. In embryonic development, insulin-producing beta cells were suspected to be engineered from the endocrine precursor cells existing in the pancreatic tissue, including the exocrine part, the ductal cells, and the endocrine part, the islet cells [49, 50]. The current consensus of scholars is that there are still certain obstacles to determine whether the potential plasticity of pancreatic tissue can serve as a source of new β cells [51, 52]. Our data also support this point of view.

Regarding the MSCs from BM and Wharton's jelly, the former expressed positive C-peptide staining on day 14 and the latter had greater staining on day 10. In addition, the BM-MSCs had differentiation potential of 4–6 passages whereas the WJ-MSCs had differentiation potential of 8–10 passages. Moreover, when at low glucose concentration, the human C-peptide levels in WJ-MSCs were higher than those of both BM-MSCs and pancreatic MCSs. Our data showed evidence similar to the literature [53, 54] that the WJ-MSCs have a certain degree of superiority than BM-MSCs.

In the *in vivo* exam, the three kinds of insulin-producing cells were transplanted into the livers of STZ-induced diabetic Sprague Dawley rats and the therapeutic effects of transplantation were evaluated. The STZ-induced diabetic rats were hyperglycemic with blood glucose results over 350 mg/dL for one week, which increased up to more than 400 mg/dL. In the Wharton's jelly MSCs treatment group, blood glucose levels returned to nearly normal levels one week after transplantation (<200 mg/dL for 6 weeks). In the BM- MSCs group, blood glucose levels also showed a significant decrease one week after transplantation (<250 mg/dL for 5 weeks). In the pancreatic IPCs group, blood glucose levels started to decrease three weeks after transplantation and were only maintained for 4 weeks at about 300 mg/dL. Based on these results, WJ-MSCs seemed to possess greater therapeutic potential than the other two cell sources.

In conjunction with the blood glucose level and the potential role of the transplanted IPCs, histological analysis after transplantation should be performed. In our previous research, we had used immunohistochemical analysis to detect the IPCs we transplanted into the STZ rat via portal vein. We had found the appearance of both human C-peptide and human nuclei labeled BM-MSCs in the liver of STZ rat 8 weeks after transplantation therapy [12]. Human C-peptide-positive cells had been detected within the liver tissue of the STZ rat 6 weeks following WJ-MSCs transplantation [11]. C-peptide expressing resected pancreas EPCs grafts were located in the STZ rat liver 9 weeks after transplantation [13]. All of the above findings suggested that the three kinds of IPCs could be functional on ameliorating hyperglycemia and capable of survival at liver after transplantation via portal vein. In this study we make efforts trying to look for whether the IPCs would engraft other places. Since groups of cells expressing both GFP and human C-peptide were detected only in the pancreas of STZ rats who received WJ-IPCs transplantation, we successfully manifested that the WJ-IPCs had homing capacity to the pancreas. Accordingly, we recommended that WJ-IPCs were better than the other two cell sources because of their superior potential for homing to the impaired pancreas tissue and providing regenerative effects in the surrounding niche.

Wharton's jelly was used by investigators in a previous study as opposed to pancreatic stem cells because it contains many times more stem cells than the pancreatic duct [8]. Additionally, WJ-MSCs can be easily isolated and expanded in culture with a higher frequency of colony-forming fibroblasts and shorter population doubling time [40]. The generation of large amounts of functional islets is an important step for the success of regeneration therapy for diabetic patients. To summarize the results of our *in vitro* experiments, it is reasonable to conclude that the WJ-MSCs were superior proliferation and differentiation potential to both the BM-MSCs and the pancreatic MSCs. In addition, Wharton's jelly stem cells are preferable to embryonic stem cells, which were thought to have excellent differentiation potential into insulin secreting cells since using them avoids the risk of forming teratomas as well as the ethical issues inherent in using embryonic stem cells.

In conclusion, our results show that WJ-MSCs can differentiate into pancreatic lineage cells *in vitro* and function as insulin-producing cells both *in vitro* and *in vivo*. These results suggest that WJ-MSCs are a promising cell source for regeneration therapy in diabetes. Further study is required to examine the curative effects of WJ-MSCs in larger diabetic animal models and to apply clinically in human beings.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Bottlenecks in the Efficient Use of Advanced Therapy Medicinal Products Based on Mesenchymal Stromal Cells

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Mesenchymal stromal cells (MSCs) have been established as promising candidate sources of universal donor cells for cell therapy due to their contributions to tissue and organ homeostasis, repair, and support by self-renewal and multidifferentiation, as well as by their anti-inflammatory, antiproliferative, immunomodulatory, trophic, and proangiogenic properties. Various diseases have been treated by MSCs in animal models. Additionally, hundreds of clinical trials related to the potential benefits of MSCs are in progress. However, although all MSCs are considered suitable to exert these functions, dissimilarities have been found among MSCs derived from different tissues. The same levels of efficacy and desired outcomes have not always been achieved in the diverse studies that have been performed thus far. Moreover, autologous MSCs can be affected by the disease status of patients, compromising their use. Therefore, collecting information regarding the characteristics of MSCs obtained from different sources and the influence of the host (patient) medical conditions on MSCs is important for assuring the safety and efficacy of cell-based therapies. This review provides relevant information regarding factors to consider for the clinical application of MSCs.

1. Introduction

MSCs are considered a heterogeneous population of non-haematopoietic progenitor cells derived from the mesodermal germ layer that have both self-renewal and multidifferentiation [1] abilities. MSCs found in virtually all postnatal organs and tissues [2] possess multifaceted features, making them promising candidate sources of donor cells for use in cell therapy and transplantation. MSCs function in the repair and support of tissues, contributing to tissue homeostasis. Although the exact origin of MSCs remains elusive, strong evidence has indicated that MSC progenitors are in the perivascular zone [3] in an environment that promotes a quiescent-resting state, ensuring homeostasis maintenance. When a tissue is damaged and the whole machinery of the organism begins to operate the body's repair mechanisms, MSCs enter the blood stream and are attracted by proinflammatory cytokines at injury areas. Thus, MSCs have been called "*guardians of inflammation*" [4]. The cytoskeleton, extracellular matrix molecules, cell contacts, adhesion ligands, and receptors are involved in the repair process [5]. Although the exact mechanisms related to the migration of MSCs into specific sites and across the endothelial cell layer remain unknown, chemokines and their receptors may play roles in this process.

Although MSC survival, permanent engraftment, and differentiation into resident cells was thought to be necessary to obtain the beneficial effects of these cells initially, clinical experience and several experiments have shown that one of the primary functions of MSCs, most likely their key function, is to secrete several bioactive molecules related to the microenvironment in which these cells are immersed.

Table 1: Summary of mesenchymal stroma cell nomenclature.

Nomenclature	Year	Authors' references
CFU-F and osteogenic stem cells	1974	Friedenstein et al. [10]
Stromal stem cells	1988	Owen and Friedenstein [16]
Mesenchymal stem cells	1991	Caplan [17]
Mesenchymal progenitor cells	1999	Dennis et al. [18]
Skeletal stem cells	2000	Bianco and Robey [19]
MAPCs and mesodermal progenitor cells	2002	Jiang et al. [20]
Multipotent mesenchymal stromal cells	2006	Dominici et al. (ISCT) [21]
Medicinal signalling cells	2010	Caplan [22]

CFU-F: colony-forming unit fibroblasts; MAPCs: multipotent adult progenitor cells; ISCT: International Society for Cell Therapy.

MSCs secrete a wide variety of proinflammatory and anti-inflammatory cytokines, chemokines, growth factors, and prostaglandins under resting and inflammatory conditions [6]. These molecules are associated with immunomodulation (indoleamine-2,3-dioxygenase (IDO), prostaglandin-E2 (PGE-2), TGF-β, HLA-G5, and HGF), antiapoptosis (VEGF, GM-CSF, TGF-β, Stanniocalcin-1, and IGF-I), angiogenesis (VEGF, MCP-1, and IGF-I), local stem and progenitor cell growth and differentiation support (SCF, Angiopoietin-1, and SDF-1), antifibrosis (HGF and bFGF), and chemoattraction (CCL2, CCL4, and CXCL12) [7]. Additionally, beneficial effects of the use of MSC conditioned media (CM) have been reported; even CM has been shown to be therapeutically better than the cells themselves [8, 9].

However, although these properties are generally attributed to all MSCs derived from different tissues, evidence from different studies has suggested that MSCs from diverse sources are not identical and do not always achieve the same efficacy levels and desired outcomes. Likewise, diverse donor conditions can affect the MSC characteristics because the environment "*niche*" in which MSCs are immersed may be affected. In this review, we will describe some of the biological characteristics of MSCs that must be considered and the effects of the disease status of donors and recipients on these characteristics.

2. Biological Characteristics

2.1. Phenotypic Profile. Since Friedenstein and colleagues first isolated a colony-forming unit fibroblast (CFU-F) from bone marrow (BM) [10], bone marrow has been widely used as a source of MSCs for many investigations and clinical trials. In addition to bone marrow, MSCs have been isolated from different tissues such as adipose tissue [11], umbilical cord blood [12], dental pulp [13], synovial liquid and amniotic fluid [14, 15]. All these tissues vary in their cellular components, signals, and factors secreted, resulting in different immediate microenvironment conditions, thus developing several physiological niches. Although isolated and long-term cultured MSCs of most tissues show similar immunophenotypic characteristics, some differences have been found among MSCs of different tissue origins according to data obtained by *in vitro* experiments. In 2006, the International Society of Cellular Therapy (ISCT) published the minimal criteria to define MSCs by nomenclature (Table 1) and by biological characteristics [10, 16–22] to allow studies from different groups to be compared and contrasted. These criteria include the following: (i) coexpression of markers such as CD73, CD90, and CD105 and a lack of expression of haematopoietic markers (CD45, CD34, and CD14) and human leucocyte antigen (HLA-DR), (ii) multipotent differentiation potential, and (iii) adherence to plastic. However, several researchers have noted that adipose-tissue-derived MSCs (AD-MSCs) express CD34 and CD54 in early passages [23] and have lower expression of CD106 and that umbilical cord blood-derived MSCs (UCB-MSCs) express CD90 and CD105 [24]. Other markers have been used in different studies, and other differences have emerged, such as VEGFR-2 (Flk-1) expression, which was significantly higher in periosteum-derived cells compared to that in adipose tissue- and muscle-derived cells, or the rate of NGFR positivity, which was much higher in muscle-derived cells compared to that in other mesenchymal tissue-derived cells [25].

Although some immunophenotypic differences have been documented, many researchers consider the fact that these differences could be due to distinct extraction methods and different culture methodologies, resulting in variations of MSC surface markers. Thus, this review aimed to further investigate markers and characteristics that are more specific to select the better sources of MSCs for clinical applications.

Likewise, expanding the cells *in vitro* is necessary to obtain the desired numbers for therapeutic approaches. Changes in the proteomic phenotype of AD-MSCs have been observed during passages [26], although no proper approaches to examine the state of cells continuously during long-term *in vitro* culture have been established. Some researchers ascribe these variations to the adaptation of cells to the environment; thus, determining the biomolecular markers that are involved in these variations is essential for obtaining a better phenotypic characterisation of these cells and thus for achieving more effective cell therapy in the future.

2.2. MSC Proliferation. The proliferative activity of MSCs is another feature that may be affected by the different origins of MSCs. The rate and persistence of MSC proliferation appear to vary between source tissues. MSCs are considered adult stem cells, and, unlike embryonic stem cells (ESCs), these

cells have a limited proliferative capacity. Physiological niches maintain adult stem cells in an undifferentiated state; however, when MSCs are cultured *in vitro*, they age, which affects their therapeutic properties, such as alterations in phenotype, differentiation potential, global gene expression patterns, miRNA profiles [27], and even chromosomal abnormalities [28], particularly after long-term culture or when cells of multiple doublings are used. Large numbers of MSCs are needed for therapeutic applications, and *in vitro* expansion is required to produce the desired MSC numbers. *In vivo*, MSCs represent 0.0001% of nucleated BM cells, and their number decreases with the age of the donor. The quantity of MSCs (CFU-Fs) among nucleated BM cells decreases with age from one MSC in 10^4 BM cells in newborns to one MSC in 10^5 cells in teenagers and to one MSC in 10^6 cells in older individuals [29]. Furthermore, MSCs from older human donors differ significantly from those from younger donors in morphology, replicative lifespan [30], doubling time, healing capacity [31], and differentiation potential. Sufficient evidence has indicated that MSCs from older donors have limited therapeutic efficacy, and some studies have suggested that the difference between preclinical and clinical findings is due to the donor age. Therefore, considering that several age-related diseases exist and that elderly patients are potential users of cell therapy, understanding the molecular and biological effects of ageing on MSCs is essential for developing safe and effective MSC-based autologous cell therapy. Meanwhile, the use of allogeneic MSCs may be a treatment option for these specific patients. As we comment below, MSCs elude allogeneic rejection, and their infusion is feasible and well tolerated, with no adverse effects [32, 33].

2.3. Differentiation Capacity. MSCs have the ability to differentiate *in vitro* into several mesenchymal lineages including adipose tissue, bone, cartilage, and muscle [15, 34, 35]. Furthermore, MSCs can differentiate into endothelial cells, neurons, and glial cells because MSCs express genes related to specific lineages rather than to those of the mesenchymal lineage [36]. Although multilineage differentiation is another minimal criterion advised by the ISCT and undoubtedly represents a fundamental property of MSCs, this ability depends primarily on the source tissue from which these cells are derived. As discussed by Sakaguchi et al. [25], who compared human MSCs isolated from bone marrow, synovium, periosteum, skeletal muscle, and adipose tissue and expanded them by similar processes, synovium-derived cells have the greatest ability for chondrogenesis; adipose- and synovium-derived cells have the greatest ability for adipogenesis; and bone marrow-, synovium-, and periosteum-derived cells have the greatest ability for osteogenesis. In another comparative analysis, UCB-MSCs showed no adipogenic differentiation capacity in contrast to BM- and AT-MSCs [37]. As discussed by Horwitz et al. [38], who used differentiated MSCs in a study to test the regeneration of damaged tissues, BM-MSCs can engraft after transplantation, differentiate to functional osteoblasts and contribute to the formation of new dense bone in children with osteogenesis imperfecta. Most likely, the microenvironment in which MSCs are transplanted directly influences their distinct differentiation pathways. New insights into the biological characteristics of MSCs are needed to achieve future therapies.

2.4. Immunomodulatory Actions. Immunomodulatory properties of MSCs and their immunoprivileged condition make these cells good candidates for use in several clinical trials related to chronic, inflammatory, and autoimmune diseases. MSCs interact with cells of the innate or adaptive immune system (T cells, B cells, NK cells, monocyte-derived dendritic cells, and neutrophils) [39, 40]. For a cell to be recognised by the immune system, the expression of major histocompatibility complex (MHC) and costimulatory molecules is necessary. MHC class I and class II human leukocyte antigens (HLAs) are master triggers of robust immunological rejection of grafts because they present antigens to cytolytic T lymphocytes (CTL) [41]. Human mesenchymal stem cells (hMSCs) are characterised by low expression of MHC class I HLAs but are constitutively negative for class II HLCs; these cells do not express costimulatory molecules such as B7-1, B7-2, CD80, CD86, CD40, and CD40L [42]. However, similar to the thymic epithelium, MSCs express the surface markers VCAM-1, ICAM-2, and LFA-3 [42, 43], which are crucial for T cell interactions. Although a T cell response should be expected, hMSCs are able to modulate the activation and proliferation of both CD4+ and CD8+ cells *in vitro* by arresting T cells in G0/G1 phase [44, 45]. Different studies have suggested that cell-cell interactions and certain soluble factors are the mechanisms used by MSCs to mediate the immune response. Factors such as IDO, TGF-β1, IFN-γ, IL-1β, TNFα, IL-6, IL-10, PGE-2, HGF, and HLA-G5 are secreted by MSCs or released after interactions with target cells. As we mentioned above, MSCs remain in a resting state, display antiapoptotic properties and maintain different cells such as haematopoietic stem cells (HSCs), thus contributing to tissue homeostasis. However, in an inflammatory environment such as that created by cytokines such as IFN-γ, TNF-α, IL-1α, and IL-1β, MSCs begin to exert their immunosuppressive effects and polarise, inhibiting the proliferation of effector cells and their production of cytokines. In this regard, IFN-γ is postulated as a "*licensing*" agent for MSC antiproliferative action. MSCs may also acquire behaviour as antigen-presenting cells (APCs) under certain concentrations of IFN-γ [46, 47]. However, no consensus regarding what concentration of IFN-γ is more necessary for MSCs to show that inhibitory or APC functions exists. Likewise, TNF-α is another proinflammatory cytokine involved in the MSC immune response, and TNF-α enhances the effect of IFN-γ [48]. IFN-γ, with or without the help of TNF-α, stimulates the production of IDO by MSCs, inhibiting the proliferation of activated T or NK cells [49] and thus enhancing the homing potential and reparative properties of these cells; however, some potential risks are associated with the role of IFN-γ [50].

Some authors have maintained that the immunomodulatory properties of MSCs are comparable [51, 52], while others have argued that MSCs of different tissue origins or species cannot have equivalent immunomodulatory properties [53, 54]. For example, MSCs from perinatal sources (umbilical cord and amniotic membrane) show a higher immunomodulatory capacity, differential gene expression profiles, and

paracrine factor secretion compared to BM-MSCs [55]. Interestingly, in 2012, Lee and colleagues found that HLA-G, a specific MHC-I antigen that is critical for maintaining the immune-tolerant state of pregnancy and that is a contributing factor to the induction of stronger immunosuppression [56], is strongly positive only in placenta-derived MSCs (PD-MSCs) in contrast to BM-MSCs and AD-MSCs, suggesting that the immunophenotype of PD-MSCs may be superior to other MSCs in terms of their immunosuppressive function [57]. Nevertheless, in another related study, BM-MSCs were more immunomodulatory than PD-MSCs [58]. Melief et al. [59] concluded that the immunomodulatory capacities of BM-MSCs and AD-MSCs are similar but that differences in cytokine secretion cause AD-MSCs to have more potent immunomodulatory effects than BM-MSCs.

A 2002 study showed that allogeneic MSCs prolonged skin graft survival in baboons [60]. Mouse MSCs have been used in related experiments; these cells use inducible nitric oxide synthase (iNOS) for immunosuppression instead of IDO. These findings indicate that MSCs differ between species [61]. Since then, several preclinical models have been used to analyse the biological effects of MSCs and their ability to modulate immune responses, considering that not all animal models mimic human diseases.

Once more, these differences could be due to isolation procedures, to culture methodology, or, more likely, to differences in the microenvironments where cells reside. These and other findings lead us to believe that determining whether these differences may be relevant for their clinical applications and whether MSCs of a particular tissue type are more appropriate for specific therapies or diseases is important.

3. Preclinical Applications

Preclinical models are essential for clinicians, researchers, and both national and international regulatory agencies to demonstrate the safety and efficacy of MSC-based therapies [62]. Because MSCs are able to exert immunomodulatory properties and to act on different immune cells both *in vitro* and *in vivo* as mentioned above, these cells have begun to be used against autoimmune diseases based on various autoimmune experimental models. Pioneer studies in experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, reported that MSCs derived from various tissue origins show efficacy against neurodegenerative disorders [63–68]. BM-MSC and UCB-MSC treatments have brought about improvements in clinical and laboratory parameters in systemic lupus erythematosus (SLE) [33, 69]. Furthermore, ameliorating effects have been observed in experimental mouse models of rheumatoid arthritis (RA) [70]. Diabetes is another autoimmune disorder in which MSCs have been employed [71–73]. Although promising results and progress have been observed in this field, the interspecies differences and contradictory experimental outcomes, as well as the inability to recreate the complete pathophysiology of some diseases, make it necessary to search for new animal models to yield comparable results.

4. Autoimmune Diseases

MSCs are being used to facilitate the engraftment of transplanted HSCs and to treat graft-versus-host disease (GVHD) after allogeneic haematopoietic stem cell transplantation (HSCT) based on their immunomodulatory properties and their ability to provide appropriate conditions; however, preclinical and clinical experiments with MSCs do not always show similar results for the prevention and treatment of GVHD. In a study using a mouse model of GVHD [74], MSCs suppressed alloantigen-induced T cell proliferation *in vitro* in a dose-dependent manner but yielded no clinical benefit regarding the incidence or severity of GVHD. Instead, when UCB-MSCs were administered in weekly doses in a xenogeneic model of GVHD, a marked decrease in human T cell proliferation was observed, and none of the mice developed GVHD. No therapeutic effect was obtained when UCB-MSCs were administered at the onset of GVHD [75]. In the same line of research, serial infusions of mouse AD-MSCs could efficiently control the lethal GVHD that occurred in recipients transplanted with haploidentical haematopoietic grafts [76]. Mixed results have also been achieved in human patients. One study found that the cotransplantation of culture-expanded MSCs and HSCs from HLA-identical sibling donors after myeloablative therapy accelerated haematopoietic engraftment [77]; however, a significant reduction of GVHD symptoms was not shown, although the incidence or severity of GVHD did not increase. Koç et al. [78] reported a positive impact of MSCs on haematopoiesis; rapid haematopoietic recovery was observed in a clinical study with breast cancer patients who received autologous HSCT together with autologous MSCs. Therapeutic effects have also been reported at the onset of GVHD, such as the case of a 9-year-old boy with severe treatment-resistant GVHD after allogeneic HSCT for acute lymphocytic leukaemia who received haploidentical MSCs derived from his mother. He showed improvement after 2 MSC administrations [79]. Similar results have been obtained in steroid-refractory GVHD pilot studies with BM-MSCs and AD-MSCs [80, 81]. Several infusions appear to be required to maintain the level of active immunomodulation by MSCs. Similarly, the expression of proinflammatory cytokines such as IFN-γ in the environment at the time of MSC administration is required by these cells to exert their immunosuppressive effect because a lack of MSC "*licensing*" can result in the absence of the desired therapeutic effect.

While evidence that MSCs are effective in combination or after HSCT in specific haematological and nonhaematological diseases has been shown, adverse reactions and risk factors intrinsic to this practice have been reported. In a pilot study, HLA-identical sibling-matched HSCs were transplanted with or without MSCs in haematological malignancy patients. Although MSCs were well tolerated and this treatment effectively prevented GVHD, six patients (60%) in the MSC group and three (20%) in the non-MSC group had 3-year disease-free survival rates of 30 and 66.7%, respectively [82]. The relapse rate in the experimental group was higher than that in the control group, suggesting that MSCs may impair the therapeutic graft-versus-leukaemia (GVL) effect.

In vitro and *in vivo* studies regarding the relationship between the immunosuppressive properties of MSCs and the stimulation of cancer growth have been performed. Mouse MSCs from BM, spleen, and thymus that were injected together with a genetically modified tumour cell vaccine could equally prevent the onset of an antitumour memory immune response, thus leading to tumour growth in normally resistant mice [83]. In another *in vivo* experiment with a murine melanoma tumour model, the authors observed that the subcutaneous injection of B16 melanoma cells led to tumour growth in allogeneic recipients only when MSCs were coinjected [84]. The functions of MSCs can be influenced by the existing microenvironment, making them acquire supportive properties towards cancer cells and decrease immune reactions [85]. Therefore, potential risks, related to the growth support and enhancement of undetected or "*resident*" cancer, do exist, and the administration of MSCs in these patients must be thoroughly evaluated.

5. Do MSCs Carry out the Patient's Disease?

One of the strategies to obtain MSCs for therapeutic purposes is an autologous approach. These cells are collected from patients by more or less invasive methods, isolated, seeded in culture under good manufacturing practice (GMP) quality standards, and reinjected into the patient. Nevertheless, when the repair mechanisms of the body are insufficient or ineffective, this treatment results in a homeostatic imbalance in the organism, producing degradation and disease and compromising the pool of endogenous cells, thus resulting in low efficacy. Some diseases provoke changes in the bone marrow microenvironment, which is one of the primary sources of MSCs, thus producing changes in the endogenous pool of MSCs and altering their biological features [86]. MSCs from patients with acute myeloid leukaemia showed abnormal biological properties, including morphological heterogeneity, limited proliferation capacity, and impaired differentiation and haematopoiesis support ability [87]. MSCs derived from patients with multiple myeloma showed impaired immune-inhibitory effects on T cells, decreasing their osteogenic potential [88]. Poor proliferation, differentiation potentials, and cytokine release defects were found in BM-MSCs derived from patients with aplastic anaemia, another haematopoietic disorder [89, 90].

Although the mechanisms remain unknown, MSCs appear to be involved in autoimmune pathologies. For instance, MSCs derived from patients with autoimmune diseases display the following altered functions. (i) MSCs from rheumatoid arthritis (RA) patients have an impaired ability to support haematopoiesis [91] and lower proliferative and clonogenic potentials [92]. (ii) MSCs from immune thrombocytopenic purpura (ITP) patients have a reduced proliferative capacity and a lower inhibitory effect on T cell proliferation compared with MSCs from healthy donors [93]. (iii) MSCs from systemic lupus erythematosus (SLE) patients display deficient growth, abnormal morphology, and upregulated telomerase activity [94, 95]. (iv) MSCs from systemic sclerosis (SSc) patients display early senescence [96]. In metabolic diseases such as diabetes, alterations in autologous MSCs have also been documented. A study using MSCs from type 2 diabetic mice showed that the number of these cells was diminished and that their proliferation and survival abilities were impaired *in vitro*. Moreover, diabetic MSC engraftment produced limited improvement in the diabetic subjects and could not produce the same therapeutic outcomes as in their nondiabetic counterparts *in vivo* [97]. Advanced glycation end products (AGEs) accumulate in the tissues of aged people, and these products are involved in diabetes and in musculoskeletal diseases. In 2005, Kume et al. [98] investigated the effect of AGEs on MSCs and showed that AGEs inhibited MSC proliferation, induced MSC apoptosis, and interfered with MSC differentiation into adipose tissue, cartilage, and bone. Another study examined type 2 diabetes-derived AD-MSCs and found that these cells had functional impairments in their multilineage potential and proliferative capacity because of prolonged exposure to high glucose concentrations [99]. We demonstrated that diabetic-derived AD-MSCs have an altered phenotype related to plasminogen activator inhibitor-1 (PAI-1) expression levels and display reduced fibrinolytic activity [100]. In this respect, our preliminary results and others suggest that the immunogenicity of MSCs could have related effects on the coagulation system [101, 102]. Thus, MSC-based therapy could lead to thrombotic events in particular recipients.

Although the possibility of healing with our own cells is extremely attractive, little is known regarding the influence of different disease states and concomitant medications on MSCs [103, 104]. Thus, although the use of autologous MSCs for cell therapy is widespread, their use in humans must be handled with extreme caution. Researching and analysing both the risks and benefits of this therapy in individual patients and for each disease state are necessary.

6. Safety and Efficacy in Clinical Trials

Several clinical trials are in progress to ensure the safety and efficacy of MSCs used as medicaments. For cell-based products, we must consider that cells are living products and that their interactions with body fluids remain unclear [100, 102, 105].

Phase I clinical trials are the first step in the investigation of a new drug and include pharmacokinetic and pharmacodynamic studies in which the patient's safety plays an essential role in the development of medicaments. The primary goal of phase II clinical trials is to provide preliminary information regarding the drug efficacy and safety supplement data obtained in phase I trials. Usually, safety evaluations are based on possible complications derived from the procedure in a time-dependent manner after the administration of the cells. Efficacy parameters focus on the improvement of clinical effects at a given time. MSC-based cell therapy is a relatively new therapeutic option for certain diseases, and data regarding the long-term monitoring of patients remain lacking. Nevertheless, the administration of MSCs is considered a feasible and safe procedure with no adverse events reported. However, the risks associated with stem cell

Table 2: Advanced therapy medicinal products (ATMPs) with a valid marketing authorisation.

Trade name	Company	Authorised by	Cell type	Indication
Carticel[b]	Genzyme (Sanofi Biosurgery)	FDA (1997)	Autologous cultured chondrocytes	For the repair of symptomatic cartilage defects of the femoral condyle (medial, lateral, or trochlea) caused by acute or repetitive trauma in patients who have had an inadequate response to a prior arthroscopic or other surgical repair procedure
Chondron[d]	Sewon Cellontech Co., Ltd.	KFDA (2001)	Autologous chondrocyte implantation	Articular cartilage defects
Articell[d]	Duplogene	KFDA (2002)	Autologous cultured chondrocytes	Articular cartilage defects
Haloderm[d]	Tego Science	KFDA (2002)	Cultured epidermal autograft	Burn wounds
Kaloderma[d]	Tego Science	KFDA (2005)	Allogeneic keratinocyte sheet	Burn wounds
JACE[e]	J-TEC (Japan Tissue Engineering Co., Ltd.)	Japan's Ministry of Health, Labour & Welfare (2007)	Autologous cultured epidermis	Burn wounds
Chondrocelect[c]	Tigenix, Belgium	EMA (2009)	Autologous cultured chondrocytes	In adults to repair damage to the cartilage in the knee
Provenge[b]	Dendreon Corporation	FDA (2010) EMA (2013)	Autologous PB-MNS activated with FAP-GM-CSF (sipuleucel-T)	In autologous cellular immunotherapy for the treatment of asymptomatic or minimally symptomatic metastatic castration-resistant (hormone refractory) prostate cancer
LaViv[b]	Fibrocell Technologies, Inc.	FDA (2011)	Autologous cultured fibroblasts (azficel-T)	Indicated for the improvement of the appearance of moderate to severe nasolabial fold wrinkles in adults
Hemacord[b]	New York Blood Center, Inc.	FDA (2011)	Allogeneic HPC, cord blood	For use in unrelated donor haematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for haematopoietic and immunological reconstitution in patients with disorders affecting the haematopoietic system that are inherited or acquired or that result from myeloablative treatment
Glybera[a]	UniQure Biopharma B.V., Netherlands	EMA (2012)	Alipogene tiparvovec	To treat adults with lipoprotein lipase deficiency who have severe or multiple attacks of pancreatitis (inflammation of the pancreas) despite maintaining a low-fat diet
Gintuit[c]	Organogenesis Incorporated	FDA (2012)	Allogeneic cultured keratinocytes and fibroblasts in bovine collagen	Allogeneic cellularized scaffold product indicated for topical (nonsubmerged) application to a surgically created vascular wound bed in the treatment of mucogingival conditions in adults
Ducord[b]	Duke University School of Medicine	FDA (2012)	Allogeneic HPC, cord blood	For use in unrelated donor haematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for haematopoietic and immunological reconstitution in patients with disorders affecting the haematopoietic system that are inherited or acquired or that result from myeloablative treatment

Table 2: Continued.

Trade name	Company	Authorised by	Cell type	Indication
JACC[e]	J-TEC (Japan Tissue Engineering Co., Ltd.)	Japan's Ministry of Health, Labour & Welfare (2012)	Autologous cultured cartilage	Articular cartilage defects
MACI[c]	Genzyme (Sanofi Biosurgery)	EMA (2013)	Matrix-induced autologous cultured chondrocytes	For implant to repair cartilage defects at the ends of the bones of the knee joint
Allocord[b]	SSM Cardinal Glennon Children's Medical Center	FDA (2013)	Allogeneic HPC, cord blood	For use in unrelated donor haematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for haematopoietic and immunological reconstitution in patients with disorders affecting the haematopoietic system that are inherited or acquired or that result from myeloablative treatment

FAP-GM-CSF: prostatic acid phosphatase granulocyte-macrophage colony-stimulating factor; HPC: haematopoietic progenitor cells; PB-MNS: peripheral blood mononuclear cells.

[a]GTMP: gene therapy medicinal product; [b]SCTMP: somatic cell therapy medicinal product; [c]TEMP: tissue-engineered medicinal product; [d]biotechnology-based product (KFDA Classification); [e]medical devices (Japan Classification).

Table 3: MSC cell-based therapies with a valid marketing authorisation.

Trade name	Company	Authorised by	Cell type	Indication
Hearticellgram*	FCB PharmiCell	KFDA (2011)	Autologous BM-derived MSCs	Treatment for postacute myocardial infarction
Cartistem*	Medipost	KFDA (2012)	Allogeneic hUCB-MSCs	Treatment of traumatic and degenerative osteoarthritis
Prochymal*	Osiris Therapeutics Inc.	Health Canada (2012) New Zealand (2012)	Allogeneic BM-MSCs	Treatment of acute GvHD children who are unresponsive to steroids

MSCs: mesenchymal stem cells; BM: bone marrow; hUCB: human umbilical cord blood; GvHD: graft-versus-host disease; KFDA: Korean Food and Drug Administration.
*SCTMP: somatic cell therapy medicinal product.

therapy [106] must be considered because these risks increase the probability of the occurrence of an adverse event. The cell source, donor origin, product manufacturing, and recipient disease status are important factors related to the safety and efficacy of the use of MSCs. In this regard, the use of bovine proteins in the medium used to culture these cells [38] and the observed formation of ectopic tissue in animal models [107, 108], as well as malignant transformation [109, 110] and immune responses, must be evaluated before wider clinical applications and registration are accepted.

7. Clinical Manufacturing of MSC-Based Medicines

With the exception of haematopoietic stem cell transplants, stem cell therapies used for the treatment of any disease are considered drugs; therefore, their development, approval, and use must be in accordance with specific standards established for such medicines nationally and internationally. MSCs are called advanced therapy medicinal products (ATMPs) and are under regulation number 1394/2007. Relating production processes and development staff, clinicians, and researchers is obligatory to achieve GMP procedures under European regulations [111, 112]. Currently, no standardised manufacturing platform exists, although most facilities employ standard release criteria to measure sterility, viability, and chromosomal stability to meet European or FDA regulations [113]. Although regulation establishes common parameters to follow, different protocols are used to isolate these cells, and the processes, plating densities, and reagents used cause the results to differ from each other. Donor selection in terms of age and disease status is another variable to consider due to known MSC donor-to-donor heterogeneity [114]. The cell source is another important factor related to the efficacy of the product. As reported previously, MSCs derived from different tissues do not always achieve the same level of efficacy. Additionally, culture media used for the production of MSCs could affect the basic characteristics of cells; thus, designing a fully defined medium free of animal and human origins is crucial.

Thus far, no MSC-based medicine product has marketing authorisation in the European Union, although four gene and cell-based products have a valid marketing authorisation awarded by the European Medicines Agency. However, since 2011, three MSC products have received marketing approval in other regions [115] (Tables 2 and 3).

The MSC field continues its upward progression, with a growing number of established companies and ongoing clinical trials, but remaining challenges must be overcome. Bottlenecks exist regarding donor selection, cells sources, isolation protocols, culture media used, open-culture systems, bioreactors, and recipient disease status. Establishing a standardised and comparable process is also crucial to ensure biological and functional equivalence between product lots.

8. Concluding Remarks and Future Perspectives

Treatments based on the use of human stem cells are novel and promising therapeutic alternatives for some diseases. Spain is at the forefront of research using such treatments, and these treatments are developed and evaluated with great scientific rigor. Currently, the use of living cells as a medicinal product is becoming realistic. Cell therapy should be safe, pure, stable and efficient. Cell-based products are more complex and depend on the physiological and genetic heterogeneity of the patient. Obtaining as much information as possible with the tools we have at our disposal is essential for ensuring the safety, reliability, quality, and effectiveness of the manufactured product. MSCs are leading the way into a new era of regenerative medicine, and their multifaceted features make them powerful candidates to become tools to treat several diseases. However, their indiscriminate use has resulted in mixed outcomes in preclinical and clinical studies. While MSCs derived from diverse tissues share some common properties, they markedly differ in terms of their differentiation abilities, growth rates, healing capacity, and gene expression profile. Similarly, the disease statuses of donors and recipients are important factors to consider when using MSCs as therapeutic agents because factors such as the MSC behaviour with body fluids and specific disease environments remain unclear. Available data suggest that some tissue-specific MSCs are more appropriate than others according to particular pathologies. Equally, some evidence has indicated that certain patient profiles are not suitable to be treated with these therapies. Thus, multiple bottlenecks for the standardisation of therapeutic protocols exist. Future well-designed clinical trials and long-term monitoring of

patients are crucial for obtaining additional information regarding the therapeutic use of MSCs.

Conflict of Interests

The authors declare that no conflict of interests regarding the publication of this paper exists.

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Review Article

Multipotent Mesenchymal Stromal Cells: Possible Culprits in Solid Tumors?

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The clinical use of bone marrow derived multipotent mesenchymal stromal cells (BM-MSCs) in different settings ranging from tissue engineering to immunotherapies has prompted investigations on the properties of these cells in a variety of other tissues. Particularly the role of MSCs in solid tumors has been the subject of many experimental approaches. While a clear phenotypical distinction of tumor associated fibroblasts (TAFs) and MSCs within the tumor microenvironment is still missing, the homing of bone marrow MSCs in tumor sites has been extensively studied. Both, tumor-promoting and tumor-inhibiting effects of BM-MSCs have been described in this context. This ambiguity requires a reappraisal of the different studies and experimental methods employed. Here, we review the current literature on tumor-promoting and tumor-inhibiting effects of BM-MSCs with a particular emphasis on their interplay with components of the immune system and also highlight a potential role of MSCs as cell of origin for certain mesenchymal tumors.

1. Introduction

Although multipotent mesenchymal stromal cells were first described in the context of regenerative medicine in the early 1970s, further research could reveal remarkable features other than their plasticity towards the osteogenic, chondrogenic, and adipogenic line [1, 2]. Particularly their immunosuppressive potential has gained widespread attention and paved the way to their application in a variety of immune disorders such as Graft-versus-Host Disease or multiple sclerosis [3, 4]. A growing body of literature in the last years has focused on a potential role of MSCs in malignancies, covering mainly two aspects: MSCs as a potential cell of origin for certain mesenchymal tumors on the one hand and the interplay of MSCs with different components of the tumor microenvironment on the other hand. These issues are of pivotal importance as many experimental oncological therapies employ MSCs as cellular vehicles that migrate to tumor sites. In order to fully grasp the interplay of MSCs with the tumor microenvironment, it is necessary to shed light on the different cells which constitute the stroma of solid tumors.

2. The Tumor Microenvironment: A Complex Niche

In 1986, Dvorak highlighted the similarities between neoplastic and inflammatory tissue, thus founding the perception of tumors as "wounds that do not heal" [5]. This comparison is based on many similarities between inflammation and carcinogenesis, which include the recruitment of a variety of immune effector cells and mesenchymal cells such as tumor associated fibroblasts [6] (see Table 1 for an overview on different components of the tumor microenvironment).

Literature of the last years has added important functional aspects to the (in earlier times primarily histological)

Table 1: Overview on cell types that are present within the tumor microenvironment (based on [7, 59]).

Cell type	Function and contribution to the tumor microenvironment	Factors which contribute to the function
Neutrophil granulocytes	(i) Remodelling of extracellular matrix (ii) Promotion of tumor growth, angiogenesis, and metastasis	(i) MMP (ii) VEGF, TGF-β
T cells	(i) Functionally compromised in the tumor microenvironment (ii) Immunosuppression	IL-10, TGF-β
NK cells	Tumor cell lysis (often immature NK cells infiltrating the tumor)	(Reduced) NKp44/NKp33 expression in tumors
Dendritic cells	Skewed towards immunosuppression (induce regulatory T cells)	TGF-β, IL-10
Tumor associated macrophages	(i) Functionally compromised in the tumor microenvironment (ii) Mostly polarized towards the M2 phenotype (iii) Inhibition of immune cell proliferation	IL-4 (induces M2 phenotype)
Mesenchymal cells/tumor associated fibroblasts	(i) Secretion of tumor growth promoting factors (ii) Remodelling of extracellular matrix	(i) TGF-β, HGF, VEGF (ii) MMP
Endothelial cells/blood vessels	(i) Secretion of VEGF (ii) Formation of new blood vessels	VEGF, PDGF-α
Tumor cells	(i) Immunosuppression (ii) ECM remodeling (iii) Cross-talk with TAF/MSCs and induction	MMP, IL-10, IL-6

description of the tumor stroma. Among the first immune cells for which functional polarizations have been reported are macrophages: The M1 and M2 subclassification refers to macrophages that have acquired different properties depending on their previous exposure to cytokines: Roughly, the M1 macrophage has been associated with a response to stimuli from Th1 cells, while the M2 subtype is being induced by IL-4 and has been ascribed to inhibit immune cell proliferation rather than eliciting an antitumor response.

Additionally, macrophages participate in restructuring the tumor extracellular matrix by the secretion of matrix metalloproteinases and growth factors (reviewed in [7]). Thus they also interact with tumor associated fibroblasts, which secrete TGF-β, SDF-1, and other growth factors both in wounds and in tumors.

While the induction of this tumor-suppressive macrophage subtype represents the most commonly accepted functional and phenotypic change of an immune cell that enters a solid tumor, other effector cells have also been demonstrated to undergo functional changes upon interaction with the tumor microenvironment: Reduction in the expression levels of activating receptors such as NKp30 and NKp46 is a consequence of NK cell and tumor cell interaction in several entities [8, 9]. The ratio of $CD56^{bright}$ $CD16^{low}$ to $CD56^{dim}$ $CD16^{+}$ cells has been found to be shifted towards the less mature, first subtype in NSCLC [8]. Beyond the impairment of NK cell effector function, the influence of the tumor microenvironment may even reprogram NK cells towards a proangiogenic phenotype [9]: NK cells could be demonstrated to secrete angiogenic factors such as VEGF or PDGF-α which was associated with a worse prognosis in certain malignancies [10].

Other immune cells such as dendritic cells have also been reported to be compromised by the tolerogenic tumor microenvironment: Being exposed to factors such as being secreted by the tumor microenvironment, dendritic cell differentiation can be arrested in an immature state and are then enabled to induce regulatory T cells by the secretion of IL-10 and TGF-β, thus further impairing the antitumor response.

In summary, for most immune cells a polarization into a tumor-suppressive and a tumor-promoting/effector function impaired phenotype has been documented and there is substantial evidence that the tumor microenvironment compromises effector functions at various levels.

Before specifically considering the interaction of fibroblasts/tumor associated MSCs with the tumor microenvironment, a further classification of these stromal cells is necessary as many publications do not present a clear phenotypic characterization but rather focus on functional properties of fibroblastoid cells, derived from tumors.

3. From Mesenchymal Stromal Cells to Tumor Associated Fibroblasts: Two Sides of One Coin?

Multipotent mesenchymal stromal cells can be isolated from a variety of tissues such as bone marrow, adipose tissue, Wharton's jelly, peripheral blood, and others [11]. Despite this plethora of origins, the phenotypic similarities between MSCs enabled the formulation of consensus definition criteria for MSCs. They consist of a set of phenotypic markers (such as CD73, CD90, and CD105) and include the capability to differentiate into osteoblasts, chondrocytes, and adipocytes [12]. While tissue MSCs from different backgrounds meet these criteria [13], it is hitherto unclear to what extent all these features are shared by other mesenchymal cells from the same tissue: Dermal fibroblasts for instance have also been shown to exhibit a trilineage differentiation potential [14]. The same seems to be true for other mesenchymal cells which share surface markers such as CD90 and CD105 with MSCs

Table 2: (Positive) Phenotypic markers and features for both MSCs and TAFs based on [15, 60–63]. Markers for which presence in both TAFs and MSCs has been demonstrated are shown in the middle while markers which have only been demonstrated in either one of the cell types are presented in the left or right column.

MSCs	Tumor associated fibroblasts
Minimal criteria for the definition of MSCs	
CD73, CD90, CD105 (according to ISCT)	
Extracellular matrix proteins	
tenascin-c, thrombospondin-1, periostin	
Adhesion molecules/lineage markers	
HCAM, VCAM-1, MCAM, LCAM, integrin-β1,	
Growth factors/hormones	
SDF-1, NPPB, FGF, VEGF, FGFR3	
Immunological markers	
HLA-ABC	
Various other markers	**Activation marker**
CD44, CD271, CD71, CD106, CD146, MSCA-1	FAP

(Table 2), [15]. Despite this overlap in phenotypic properties, more recent studies could identify markers that are able to separate MSCs from fibroblasts such as CD106 which displays specific expression on MSCs and is absent on their fibroblast counterparts [16].

With regard to the tumor microenvironment, a comprehensive characterization of TAFs from different cancer entities aiming at the establishment of marker to discern each cell type is still missing. Spaeth et al. could demonstrate that the coculture of MSCs with supernatant from tumor cells could induce activation markers (FAP, TSP1, and α-SMA) that are typical for TAFs [17]. However, this quantitative difference in expression of TAF-associated proteins is not sufficient to qualify the aforementioned markers as a specific marker for either TAFs or MSCs. Paunescu et al. have systematically compared the expression of MSC marker molecules (such as CD44, CD90, and CD73) between MSCs, TAFs, skin fibroblasts, and HDFa and found no differences in the expression levels of these molecules [18]. Moreover, other less established MSC markers such as vimentin were also found to be commonly expressed in all fibroblastoid cell types [19].

Consistent with the results of Osonoi et al., in this and other studies, TAFs were also shown to display plasticity towards the osteogenic line.

The sole functional property distinguishing TAFs and MSCs was a higher proliferative capacity and cytokine production of TAFs when compared to BM-MSCs [19].

In absence of a single surface marker to discern TAFs and MSCs, gene expression profiling may be the only way to pinpoint the differences between MSCs and fibroblasts from cancer [20].

Given this dearth of specific markers, further evidence is needed to clarify whether both cell types are essentially identical or coexisting cell types within the tumor stroma. In the following passage we focus on studies which specifically examine bone marrow derived MSCs or MSCs isolated from tumors.

4. MSCs and Their Interplay with Components of the Tumor Microenvironment

Several aspects of MSC biology have been examined in recent literature: One approach aims at assessing the effect of tumors BM-MSCs after coinjection with subcutaneous or orthotopic xenograft tumors. The careful examination of changes in tumor growth pattern and in its microenvironmental structure after exposure to MSCs is pivotal given the numerous therapeutic studies that use MSCs for tissue regenerative and other purposes.

Another class of studies has characterized MSCs or tumor associated fibroblasts that were isolated directly from primary tumor tissue and is mainly dedicated to studying the functional effects of tumor derived MSCs on immune cells.

5. The Immunomodulatory Role of MSCs and TAFs in the Context of Solid Tumors

A functional feature which has fuelled immunological and oncological research is the immunosuppressive property of BM-MSCs. They are capable of inhibiting the proliferation of T cells in PBMC (peripheral blood mononuclear cells) preparations *in vitro* and *in vivo*.

At a more detailed level, this property expands to almost all effector cells of the peripheral blood such as T cells, B cells, and NK cells that are inhibited not only in their proliferative capacity, but also in cytolysis and antibody production.

This immunosuppression at a cellular level is widely considered to be the functional basis for the systemic effects, for example, in Graft-versus-Host Disease. This property involves several soluble factors such as galectin-3, galectin-9, indoleamine 2,3-dioxgynease (IDO), IL-10, and HLA-G [21].

With regard to the neoplastic context, the infiltration of immune cells to solid tumors is a well-described phenomenon [8].

There is growing evidence that T-MSCs share these antiproliferative and immunosuppressive functions with their bone marrow counterparts: In an *in vitro* study in human gliomas, Ochs et al. could show that MSC-like pericytes display inhibitory functions on $CD4^+$ T cells similar to BM-MSCs [22]. This effect was found to be mediated by prostaglandin-E2 and HGF which have also been implicated in the immunosuppression exerted by BM-MSCs.

More recently, the glioma promoting effect of pericytes has been validated in a xenograft model of this disease, supporting the notion that these mesenchymal cells can switch from a tumor-suppressive phenotype to a tumor-promoting one [23].

Notably, the antiproliferative effect of MSCs also affects microglia cells which represent the quantitatively most important immune cell population of the brain. Proliferation of these cells is impeded by a mechanism that involves tumor necrosis factor α (TNF-α) [24].

Table 3: Immunomodulation by MSCs and TAFs.

Tumor entity	Effects observed	Effects mediated by	Literature
Cervical cancer	Downregulation of HLA-I in cervical cancer by T-MSCs cell lines and reduced cytolysis by CTL-cells	IL-10	[26]
Melanoma	Impairment of NK cell answer against melanoma cells by reduced upregulation of NKp44, NKp33, and DNAMI after exposure to T-MSCs	(i) Reduction of NKp44 and expression (mediated by PGE2) (ii) Reduction of DNAMI expression (depending on cell to cell contact)	[64, 65]
Pancreatic cancer	Depletion of arginine renders tumor infiltrating T cells dysfunctional	Expression of ARG2 and arginine depletion	[66]
NSCLC	CD3/CD28 depending activation of T cells	IL-6	[67]
Follicular lymphoma	MSCs from follicular lymphoma patients display increased recruitment of TAM and a distinct gene expression profile	Overexpression of CCL2	[68]

Not only MSCs isolated from brain tumors but also MSCs derived from pediatric malignancies and from colorectal carcinomas display immunosuppressive properties and are able to downregulate activating NK cell receptors and impair the tumor lysis by NK cells *in vitro* [22, 25].

Montesinos et al. have drawn a direct comparison between MSCs from nonneoplastic cervical tissue and cervical cancer demonstrating an identical marker profile of both MSC types yet with distinct functional properties: Production of the immunosuppressive interleukin IL-10 was markedly increased in tumor associated MSCs, underpinning a role in establishing an immunosilenced, quiescent niche [26].

To summarize, the majority of publications state a tumor-promoting effect by suppression of immune effector cells. Hereby, the factors which have been identified as mediators of these effects are by and large the same as the ones implicated in BM-MSC immunosuppression (i.e., IDO, PGE2, and others).

Few publications contrast with these observations; Barnas et al. found that TAFs could induce CD3/CD28 depending activation of T cells and could not confirm an inhibition of these effector cells [27].

Taking the results of all these functional studies together, inhibitory effects on various immune effector cells have been demonstrated by a number of studies (Table 3).

The abundance of these publications however needs to be taken with a grain of salt: Very few experimental designs aim at a side-by-side comparison of tumor derived MSCs with fibroblasts or MSCs from adjacent healthy tissue. Given the fact that also fibroblasts from different other tissues seem to share the antiproliferative property on immune cells [15], it is questionable whether the sole measurement of effector cell proliferation or receptor expression status without an adequate control population is able to faithfully recapitulate the situation in the tumor. While the mechanism of immunosuppression is well documented, both for T-MSCs and for BM-MSCs, a comprehensive comparison with other fibroblastoid cells from nonneoplastic tissue is still missing.

6. The Homing of BM-MSCs in Solid Tumors

Aside from tissue MSCs that are already present at sites of tumorigenesis and that may undergo a differentiation to TAFs, there is substantial evidence that MSCs from the bone marrow may also home in the tumor thus contributing to the tumor stroma: This ability of MSCs has been addressed by a number of studies aiming at the therapeutic use of this property ([28], also reviewed in [29]).

A seminal study by Quante et al. could show that in the setting of inflammatory gastric cancer about 20% of TAFs originate from bone marrow MSCs [30]. The chemoattraction of BM-MSCs to the tumor in these studies was mainly governed by TGF-β and SDF-α, factors which have previously been shown to be secreted by various components of the tumor microenvironment [6]. By gene expression profiling, higher levels of inflammation-associated genes were found to be expressed in these bone marrow derived TAFs than by their bone marrow counterparts.

The exact proportion of mesenchymal cells in tumors that originate from the bone marrow may vary: In a study of ovarian cancer as much as 60–70% of the stroma could be traced back to BM-MSCs [31].

Notably, the majority of publications (for an overview please see Table 4) that could demonstrate a migration of MSCs to the tumor are conducted in adult cancers and carcinomas with a generally high proportion of mesenchymal cells (e.g., pancreatic cancer). The migration pattern of BM-MSCs to, for instance, pediatric, neoplasias remains largely understudied.

When trying to dissect the mechanism by which BM-MSCs are attracted to the tumor, most of the studies identify inflammatory cytokines as important mediators (such as SDF-1 or CXCR6). Hence the same molecules which have been identified as mediators of immune cell attraction to neoplasms are also identified as major protagonists in the context of BM-MSC homing. However, this may also be an effect of focusing the analysis to a set of well-documented factors (more comprehensive proteomic analyses from tumor supernatants are needed to identify other factors that may mediate the migration).

Table 4: Overview on homing mechanisms of MSCs and MSC effect on tumor growth.

Entity	Experimental design	Effects observed	Literature
NSCLC	*In vitro* coculture study of MSCs and tumor cells	Interaction of MIF with CXCR4/SDF-1 contributes to MSCs homing	[69]
Melanoma	*In vivo* homing of cytosine deaminase expressing MSCs in subcutaneous melanoma	MSCs abrogate tumor growth by TNF-α production	[69, 70]
Breast cancer	(i) *In vitro* and *in vivo* migration of breast cancer cells to the bone marrow being facilitated by BM-MSC (ii) Coinjection of BM-MSCs and breast cancer cells	Tac1 mediated entry of breast cancer cells to the bone marrow	[38]
Neuroblastoma	*In vitro* migration of MSCs towards neuroblastoma cell lines	Migration of MSCs depending on uPA expression	[71]
Glioblastoma/brainstem glioma	(i) *In vivo* homing of MSCs in GL216 glioma model	(i) Presence of MSCs in the GL216 glioma model validated the homing process, change of the phenotype of tumor cells due to MSC influence; CXCR4 and CXCR6 contribute to the homing of MSCs	[72]
	(ii) *In vitro* migration of MSCs towards GBM cell lines	(ii) *In vitro* migration was mediated by HGF	[73]
Mesothelioma	*In vivo* homing of TNF-α overexpressing MSCs in intraperitoneal mesothelioma	*In vitro* and *in vivo* induction of apoptosis in mesothelioma cells	[74]
Hepatocellular carcinoma	(i) *In vivo* homing of MSCs in HCC	(i) MSCs primed with AMF displayed increased migratory capability to HCC and reduced MMP2 expression	[75]
	(ii) Coculture of MSCs and HCC cell lines	(ii) Increased invasiveness of HCC due to CCL5	[76]
Multiple myeloma	Decreased survival of mice after MSCs and multiple myeloma cell infusion	CCL25 production by MM cells as a chemoattractant	[77]
Gastric cancer	Recruitment of BM-MSCs to gastric cancer	CXCR4/SDF-1 axis	[30]
Pancreatic cancer	Transplantation of bone marrow (BM) cells into sublethally irradiated SCID mice and subcutaneous transplantation of a pancreatic cancer cell line; assessment of stromal cell contribution by BM-MSCs	High frequency of BM-derived myofibroblasts in the tumor stroma	[78]

Teo et al. have shed more detailed light on the migratory mechanism by which MSCs overcome the endothelial barrier in inflammatory and cancer microenvironments: Similar to leukocytes, these cells are able to perform para- and transcellular diapedesis from the blood vessel lumen to the tumor [32].

Even in brainstem glioma models, intravenous administration of TRAIL-expressing MSCs resulted in increased apoptosis in the tumors which correlated with a significantly increased survival [33], indicating that even the blood brain barrier can be overcome. The systematic exposure of adipose tissue derived MSCs to laminin, fibronectin, and glioma-conditioned media was able to increase to rate of MSCs homing in a rodent model of glioblastoma [34].

7. Paracrine Effects of BM-MSCs and Their Role in Extracellular Matrix Remodeling within Cancers

The establishment of different homing mechanisms of MSCs in tumors prompts the question about the role of these cells after having infiltrated the tumor.

In the case of breast cancer, otherwise weakly metastatic tumor cells greatly increased their metastatic potential when stimulated by MSCs. Mechanistically, the secretion of CCL5 by MSCs seemed to be a crucial factor in this process. Notably, the effect relied on the constant production of this chemokine and was reversible when BM-MSCs and tumor cells were separated after short, initial priming.

In keeping with this finding in breast cancer, Xu et al. could demonstrate that the frequency of metastases in a human osteosarcoma model is increased, when they injected MSCs intravenously after xenografting the tumor [35, 36]. Here again, CCL5 was at least partly responsible for this effect. Another mechanism by which MSCs may increase tumorigenesis of breast cancer cells is the induction of lysyl oxidase which was shown to enhance the metastatic potential of breast cancer cells in xenografts [37].

Particularly when it comes to bone marrow metastases of breast cancer, MSCs could exert detrimental effects as they are able to promote the transmigration of breast cancer cells over endothelia *in vitro* [38].

Another link between tumor progression and MSCs was established in a model of hepatocellular carcinoma (HCC), in which the tumor growth promoting effect was strongly

dependent on the presence of TGF-β secreted by MSCs [39]. The important role of the cytokine TGF-β in the tumorigenic effect of MSCs is further highlighted by experiments from Shangguan et al. work: By transducing MSCs with activin membrane-bound inhibitor, a TGF-β receptor with inactive cytoplasmatic domain, a repression of the TGF-β axis could be achieved and the tumor protective properties of MSCs in a breast cancer model could be abrogated [40].

It is remarkable that these studies seem to converge on a relatively small set of molecules that have previously been studied in either the inflammatory context or the context of chemoattraction/immunosilencing in the tumor microenvironment. Here again, it is not clear whether the set of factors which has been examined is limited and other substances may also play a role, or whether the inflammation-related chemokines (such as CCL5) just play ubiquitous role in chemoattraction of multiple cells.

Apart from providing growth stimuli by paracrine effects, the remodeling of the extracellular matrix is another aspect by which mesenchymal cells are able to facilitate tumor progression: MMP 13 (matrix metalloproteinase 13) has been shown to promote cancer cell invasion *in vitro* and is overexpressed in mesenchymal stem cell like myofibroblasts in solid tumors [41].

A series of studies has attempted to mimic the low oxygen tension in solid tumors, thus by exposing BM-MSCs to low oxygen tensions: Potier et al. could demonstrate that temporary hypoxia of MSCs lead to a twofold increase in the secretion of vascular endothelial growth factor (VEGF), whereas the plasticity of MSCs towards the adipogenic and osteogenic line is reduced under hypoxia as it was shown by Holzwarth and coworkers [42, 43]. A potential role of VEGF produced by tumor-MSCs is supported by an *in vivo* study from Suzuki et al. work, showing that an increased rate in metastasis in Lewis lung cancer model is related to an MSC induced neovascularization in these tumors [44].

Taken together, there is strong evidence that MSCs are able to migrate to solid tumors by the help of chemoattractants. It remains a largely unsolved question to what extent BM-MSCs quantitatively contribute to the stroma and what other tissues are involved in providing cellular support for the tumor mesenchyme.

A relatively recent concept proposes distinguishing between two MSC types (MSC1 and MSC2) which, in close analogy to the macrophage type 1 or type 2, represent a physiological, nontumor propagating phenotype (MSC1) and a tumor-promoting phenotype (MSC2). Experimentally, the induction of both subtypes could be achieved by the stimulation of Toll-like receptors 3 and 4 (TLR3, TLR4). The classification into the two phenotypes is mainly based on a distinct cytokine profile which includes an overexpression of TGF-β and its downstream effectors SMAD3 and SMAD4 [45, 46]. Although first published only in the *in vitro* context *in vivo* experiments confirm the different functions of MSC1 and MSC2, further evidence is needed to confirm the pathological role of this polarization and to validate the existence of both subtypes in tumors [45, 47].

In summary, there are manifold mechanisms by which MSCs seem to exert their protumorigenic effect. They include (a) an inhibition of immune cells that are attracted to tumors as sites of chronic inflammation, (b) an induction of neovascularization that can promote tumor spread, (c) a transdifferentiation to myofibroblasts that contribute to stroma niche, (d) and lastly the remodeling of the extracellular matrix with the help of, for example, matrix metalloproteinases (as highlighted in Figure 1).

8. Evidence for Tumor-Inhibiting Properties of MSCs

Although the onus of proof points towards MSCs as tumor propagating and not tumor-inhibiting cells, the effect of MSCs may be context-depending. In fact, there are settings in which MSCs may abrogate tumor growth: When being injected into rat gliomas, MSCs are able to increase the therapeutic benefit of an immunotherapy with IFN-γ. This could be correlated to a stronger, antitumoral $CD8^+$ T cell response against tumor cells [48]. Likewise, the same therapy enhancing effects of MSCs, when being coadministered with cisplatin, could be shown in a melanoma model [49].

In Kaposi's sarcoma, cell-cell contact was necessary for MSCs to slow down tumor growth. Akt kinase was implicated in this process as its phosphorylation in cancer cells decreases upon coculture with MSCs [32]. Similar observations could be made in pancreatic cancer where the administration of MSCs retarded tumor growth [50].

In pancreatic cancer, the inhibition of tumor growth was potentiated when transducing MSCs with IFN-β, confirming their suitability as a vector for immunotherapy [50]. Although these findings of a tumor inhibition by MSCs are conflicting in the case of, for example, glioblastoma, part of the divergence may be explained by different experimental settings: The choice of the *in vitro* and *in vivo* models may have a strong impact on the interaction between MSCs and the respective cancer. Thus, conducting more comparative studies using the same models and similar experimental conditions may help to reconcile these contradictions.

9. Multipotent Mesenchymal Stromal Cells as Possible Cells of Origin for Mesenchymal Tumors?

MSCs that are isolated from neoplastic tissues are typically considered to be devoid of the genetic aberrations that characterize the respective cancer [51–53].

Nonetheless there is increasing evidence that MSCs are able to serve as progenitor cells for certain, mainly soft tissue, tumors: The experimental silencing of the Ewing sarcoma specific EWS-FLI1 fusion transcript could partially restore the adipogenic differentiation potential of an ES tumor cell line, a property which the tumor cells do not display under native conditions. On a transcriptomic level, the expression profiles of these EWS-FLI1 silenced cells show a high degree of similarity to the gene expression profiles of MSCs [54].

Along a similar line, Tanaka et al. could detect an abundance of mesenchymal progenitors in the embryonic superficial zone of mouse that could give rise to Ewing

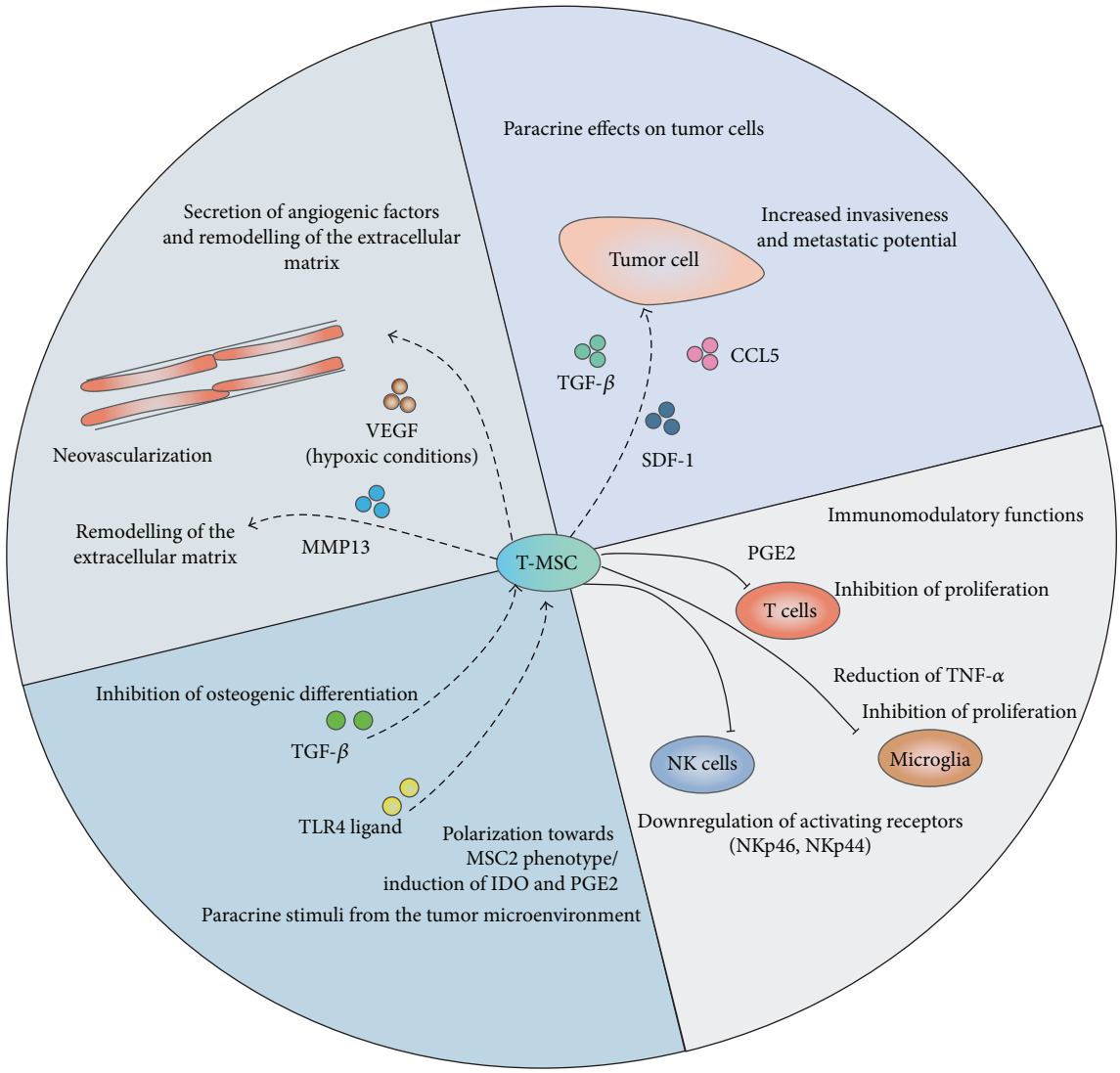

Figure 1: Interplay between T-MSC and the tumor microenvironment.

sarcomas when transduced with the EWS-FLI1 fusion transcript [55].

Aside from Ewing sarcomas, the tumorigenesis of other sarcomas has also been linked to the presence of MSCs: MSCs from a p53 −/− background can serve as cell of origin for leiomyosarcoma and osteosarcoma [56].

Taken these evidences together, although MSCs within solid tumors may preserve their cytogenetic integrity, the similarity between the aforementioned tumors and MSCs at least indirectly points to MSCs as a potential paternal cell of origin of mesenchymal tumors such as Ewing sarcoma.

Table 5 synoptically catalogues entities in which tumor propagating or tumor-inhibiting properties of MSCs could be stated or for which MSCs have been implied as a putative cell of origin.

10. Conclusions

Although MSCs from the bone marrow and other "classical" sources have been characterized extensively, the phenotypic and functional properties of MSCs from tumors are poorly understood. This is in part due to the hitherto unclear distinction of these cells from tumor associated fibroblasts which share phenotypic markers and may also exert similar functions.

While the identity of tumor derived MSCs remains controversial and the number of publications that refer to tumor derived MSC directly remains small, a plethora of experiments studies the interaction of BM or T-MSCs and tumor cells both, *in vitro* and *in vivo*. The immunosuppressive function of both types of MSCs has been validated extensively and the cytokines which are implicated into mediating the effect seem to be identical between different MSC types.

The number of publications reporting a protumorigenic role of BM-MSCs/T-MSCs outweighs the ones which show antitumorigenic effects. Furthermore, the latter often refer to genetically engineered MSCs or combination of MSCs with an additional therapeutic agent and as such do not consider the native situation [57].

Table 5: Studies in which MSCs have been implicated as cell of origin for specific tumors or have been shown to display protumorigenic or antitumorigenic effects.

MSCs as possible cell of origin	Tumor propagating effects of MSCs	Tumor inhibiting effects of MSCs
Ewing sarcoma [79]	Breast cancer [35]	Hepatoma [79]
Osteosarcoma [80]	Ovarian cancer [80]	Kaposi's sarcoma [81]
Leiomyosarcoma [80]	Head and neck squamous cancer [82]	Glioblastoma [58]
Synovial sarcoma [83]	Colon cancer [84]	Pancreatic carcinoma [50]
	Osteosarcoma [85]	Glioblastoma [86]
	Melanoma [87]	
	Hepatocellular carcinoma [88]	
	Glioblastoma [58]	

It is thus conceivable that the divergent findings on the role of MSCs in tumors may partly be due to the different settings that were used for the experiments. Moreover, the source of MSCs may influence their effect on tumor growth: Akimoto et al. demonstrated that adipose tissue derived MSCs could promote glioblastoma growth *in vitro* and *in vivo* while umbilical cord-blood derived cells inhibited the tumor progression [58].

It is noteworthy that in a given tumor entity only very few conflicting reports on tumor-inhibiting and tumor propagating effects of MSCs have been shown. This may hint at MSC-effects that are depending not only on the origin of the MSCs but also on the entity and context that is studied. To resolve the role of MSCs in tumorigenesis, more comparative examinations using identical settings between different entities are needed.

On the basis of these investigations, a final judgment on the role of MSCs may possibly be achieved; this would be highly desirable given the increasing number of clinical trials banking on the therapeutic use of BM-MSCs.

Abbreviations

α-SMA: α-smooth muscle actin
AMF: Autocrine motility factor
BM-MSC: Bone marrow derived multipotent mesenchymal stromal cell
CCL5: Chemokine ligand 5
DNAMI: DNAX accessory molecule-1
FAP: Fibroblast activation protein
FLI1: Friend leukemia integration 1
HCC: Hepatocellular carcinoma
HGF: Hepatocyte growth factor
IDO: Indoleamine 2,3-dioxgynease
IFN-β: Interferon β
IL: Interleukin
ISCT: International Society for Cellular Therapy
MIF: Macrophage inhibitory factor
MM: Multiple myeloma
MSC: Multipotent mesenchymal stromal cell
MSCA-1: Mesenchymal stem cell antigen-1
NPPB: Brain natriuretic peptide
NSCLC: Nonsmall cell lung cancer
SDF-1: Stromal derived factor 1
SMAD3: Mothers against DPP homologue 3
SMAD4: Mothers against DPP homologue 4
SSEA: Stage specific embryonic antigen A
TAC1: Protachykinin 1
TGF-β: Transforming growth factor β
TLR: Toll-like receptor
TRAIL: TNF-related apoptosis inducing ligand
TNF-α: Tumor necrosis factor α
uPA: Urokinase plasminogen activator
VCAM: Vascular cell adhesion protein-1
VEGF: Vasoendothelial growth factor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Modulation of Hyaluronan Synthesis by the Interaction between Mesenchymal Stem Cells and Osteoarthritic Chondrocytes

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Bone marrow mesenchymal stem cells (BM-MSCs) are considered a good source for cellular therapy in cartilage repair. But, their potential to repair the extracellular matrix, in an osteoarthritic environment, is still controversial. In osteoarthritis (OA), anti-inflammatory action and extracellular matrix production are important steps for cartilage healing. This study examined the interaction of BM-MSC and OA-chondrocyte on the production of hyaluronan and inflammatory cytokines in a Transwell system. We compared cocultured BM-MSCs and OA-chondrocytes with the individually cultured controls (monocultures). There was a decrease in BM-MSCs cell count in coculture with OA-chondrocytes when compared to BM-MSCs alone. In monoculture, BM-MSCs produced higher amounts of hyaluronan than OA-chondrocytes and coculture of BM-MSCs with OA-chondrocytes increased hyaluronan production per cell. Hyaluronan synthase-1 mRNA expression was upregulated in BM-MSCs after coculture with OA-chondrocytes, whereas hyaluronidase-1 was downregulated. After coculture, lower IL-6 levels were detected in BM-MSCs compared with OA-chondrocytes. These results indicate that, in response to coculture with OA-chondrocytes, BM-MSCs change their behavior by increasing production of hyaluronan and decreasing inflammatory cytokines. Our results indicate that BM-MSCs *per se* could be a potential tool for OA regenerative therapy, exerting short-term effects on the local microenvironment even when cell:cell contact is not occurring.

1. Introduction

Osteoarthritis (OA) is a pathology accompanied by an increased secretion of inflammatory cytokines and proteolytic molecules into the surrounding tissue, leading to extracellular matrix degeneration and functional impairment [1]. The capacity of adult chondrocytes to maintain cartilage homeostasis declines with age, with loss of the ability to secrete the extracellular matrix components responsible for the characteristic viscoelastic properties of the cartilage [2]. Hyaluronan and aggrecan act as the major aggregating factors for collagen, proteoglycans, and water, playing a key role in the maintenance of the cartilage structure and the ability to resist to compressive loads [3, 4].

Hyaluronan is a glycosaminoglycan composed of repeated disaccharide units synthesized by hyaluronan synthases (HASs), which are membrane-bound enzymes. There are three isoforms in humans, HAS-1, HAS-2, and HAS-3, which produce hyaluronan molecules of different molecular sizes. HAS-1 and HAS-2 produce higher molecular weight hyaluronan molecules ($>2 \times 10^6$ Da) [5, 6]. High molecular weight hyaluronan has been described as an anti-inflammatory and immunosuppressive molecule, whereas low hyaluronan fragments exhibit immunostimulatory and proinflammatory effects [7].

Degradation of hyaluronan is regulated by hyaluronidases. There are six hyaluronidase-like sequences in the human genome [7]; however, only three hyaluronidases (HYAL-1, HYAL-2, and HYAL-3) have been described in cartilage [8]. Each enzyme acts upon molecules of different molecular weight paving the way for hyaluronan turnover in

the cartilage. HYAL-1 degrades hyaluronan of all molecular weights to smaller oligomers. HYAL-2 cleaves only high and intermediate molecular weight hyaluronan yielding products of approximately 20 kDa, while little is known about HYAL-3 enzymatic activity [7, 9]. Of the three hyaluronidase genes, HYAL-2 is the most expressed in normal chondrocytes. Currently, there are indications that in OA there are lower hyaluronan levels and with altered molecular weight and that hyaluronidases are upregulated in response to inflammatory cytokines [10–12].

Inflammation has been described as an important factor in the development and progression of OA. The main proinflammatory cytokines described in the pathophysiology of OA are interleukin- (IL-) 1 beta, TNF, IL-6, and also IL-8 [13]. These cytokines contribute to the pathogenesis of OA through several mechanisms leading to a shift in chondrocytes phenotype. In an inflammatory environment chondrocytes become activated and increase further the expression of proinflammatory cytokines and factors involved in tissue catabolism, namely, matrix metalloproteinases and other proteolytic enzymes, which degrade hyaluronan, aggrecan, collagen, and fibromodulin. Such fragments of matrix components, in turn, also help maintain the production of inflammatory cytokines [1, 14]. Alternative therapies for cartilage regeneration in OA should ideally reduce inflammation and promote tissue remodeling. In this context, the use of mesenchymal stem cells (MSCs) has been pointed out as an interesting therapeutic option [14, 15], due to their distinct immunomodulatory, anti-inflammatory, and regenerative properties [16, 17]. MSCs anti-inflammatory properties might also be able to change chondrocytes phenotype, decreasing production of inflammatory molecules and favoring the renewal of extracellular components.

Indeed, interaction between MSCs and chondrocytes has been studied *in vitro*, especially in the context of cartilage development, evidencing a role of MSCs in forming cartilage tissue [18–20]. However, the effects of MSCs on OA-chondrocytes and on their capacity to repair the extracellular matrix have not yet been fully examined. Likewise, the potential effect of OA-chondrocytes on MSCs has so far been overlooked. *In vitro* coculture of MSCs and OA-chondrocytes represents a powerful approach to distinguish the contribution of each cell type and their interaction. Using cells from the same patients, we proposed to investigate the effects caused by the interaction with no physical contact between MSCs and OA-chondrocytes on the secretion of inflammatory markers and on hyaluronan synthesis.

2. Materials and Methods

2.1. Culture and Isolation of Human Bone Marrow Stem Cell and Chondrocytes. Both OA-chondrocytes and BM-MSCs were obtained from six patients undergoing total knee replacement (TKR) surgery. All patients were women (ages 63–80 years; average age: 70 years) with Grade III or IV knee OA according to the Kellgren and Lawrence classification [21]. Articular cartilage and bone marrow were harvested from the distal femur during TKR procedure. The study was carried out in full accordance with local ethical guidelines (CEP/Einstein 10/1268 Hospital Israelita Albert Einstein; CAAE: 0006.0.028.000-10) and samples were collected after obtaining written informed consent from all donors.

For isolation of chondrocytes, slices of OA knee cartilage from each donor were separately incubated in 0.25% type I collagenase in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO), overnight at 37°C in 5% CO_2. The cells were then seeded onto tissue culture flasks for expansion and maintained as subconfluent monolayers in DMEM with low glucose (DMEM-LG) supplemented with 1 mM of *L*-glutamine, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution (Gibco/Life Technologies, Carlsbad, CA).

A small volume of bone marrow was drawn from the distal femur to obtain BM-MSCs [22] and diluted in equal volume of phosphate buffered saline (PBS). The cells were then layered over Ficoll (density, 1.03 to 1.12 g/mL; GE) and centrifuged at 500 g for 30 minutes. Mononuclear cells were collected, seeded onto tissue flasks, and cultivated with DMEM-LG supplemented with 15% fetal bovine serum, 1 mM of *L*-glutamine, and 1% antibiotic-antimycotic solution (Gibco/Life Technologies). All incubations occurred in a 5% CO_2 atmosphere at 37°C. Medium was replaced 3 times a week until cells reached confluence. At 80% confluence cells were harvested with a trypsin/EDTA solution (0.25% trypsin, 4 mM EDTA; Gibco/Life Technologies) and seeded onto new flasks.

BM-MSCs were expanded until the fifth passage and analyzed by flow cytometry to determine the expression profile of stem cell markers as defined by the International Society of Cell Therapy. All BM-MSCs samples expressed CD90, CD73, and CD105 on at least 95% of all cells with very low (or absent) expression of CD45, CD34, CD14, and HLA-DR. Differentiation of BM-MSCs into three cell types (adipocytes, osteocytes, and chondrocytes) was successfully achieved after culture with specific media (StemPro Adipogenesis, Chondrogenesis and Osteogenesis Differentiation Kit, Gibco/Life Technologies) and confirmation after specific staining with Oil Red for adipocytes, Alcian Blue for chondrocytes, and Alizarin Red S for osteocytes.

2.2. Coculture of BM-MSCs and OA-Chondrocytes. Cocultures ($n = 6$) were performed by seeding the paired BM-MSCs and OA-chondrocytes at a 1 : 1 cell ratio (50,000 cells each) from each donor. BM-MSCs were seeded onto the lower chamber of a 6-well plate and OA-chondrocytes onto Millicell hanging cell culture inserts (0.4 μm pore size; Millipore, Billerica, MA, USA) in 5 mL of DMEM-LG supplemented with 10% FBS, 1 mM of *L*-glutamine, and 1% antibiotic-antimycotic solution. Controls were monocultures of BM-MSCs and OA-chondrocytes (50,000 cells each, 5 mL of medium). On days 3 and 6 both monocultures and cocultures were detached with trypsin-EDTA solution. Viable cells were counted using the Trypan blue exclusion technique using a Neubauer chamber. Total RNA from the cells harvested on both days 3 and 6 was extracted for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis and culture supernatants were stored at −80°C.

2.3. Hyaluronan Measurement. Hyaluronan measurement was performed using a highly specific enzyme-linked immunosorbent assay- (ELISA-) like fluorometric method [23]. Supernatants were boiled for 30 min in order to inactivate all proteolytic activity. Boiled sample triplicates and hyaluronan standards (ranging from 0 to 500 mg/L) were incubated in plates coated with biotinylated hyaluronan-binding protein, followed by a washing process, adding of europium-marked streptavidin, and an enhancement solution. Final fluorescence was measured in a fluorometer (Perkin-Elmer Life Sciences-Wallac Oy). Individual cell numbers were used to normalize the absolute amounts of hyaluronan of each sample, including when coculture was performed. In this last case we considered the sum of both cells types added in the coculture. Thus, data are presented as mean of hyaluronan concentration in pg/mL per cell (pg/mL/cell).

2.4. Inflammatory Cytokine Analysis. The concentrations of inflammatory molecules in the culture supernatants were simultaneously evaluated using the Cytokine Beads Array Kit (*Human Inflammation*, IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70) (BD Biosciences) by flow cytometry (FACSAria, BD Biosciences, San Jose, CA) following the manufacturer's instructions and analyzed with FlowJo (TreeStar, Ashland, OR) and BD CBA software. Concentration was normalized by cell count in each culture group and the relative production of inflammatory cytokines was expressed as ng/mL/cell.

2.5. Gene Expression Analysis. Relative quantification of mRNA expression of hyaluronan enzymes, of extracellular molecules, and of inflammatory cytokines was performed using qRT-PCR. Total RNA was extracted with TriZol (Life Technologies) and a reverse transcriptase reaction (QuantiTect Reverse Transcription Kit, QIAGEN) was performed. qRT-PCR was carried out using the ABI7500 thermocycler (Applied Biosystems, Carlsbad, CA) and the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA) for hyaluronan enzymes and extracellular molecules, according to the manufacturer's recommendations. Primer sequences are shown in Table 1. Expression of target genes was normalized by β-actin mRNA levels. The level of expression was then calculated as $2^{-\Delta\Delta Ct}$ and expressed as the mean. The results are presented as mean fold change relative to a calibration sample (Reference RNA for Real-Time qPCR, #636690, Clontech, Mountain View, CA, USA). For coculture analysis, fold change is presented as gene expression relative to each BM-MSC or chondrocyte monoculture.

2.6. Statistical Analysis. All data analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA). Statistically significant differences per cell in hyaluronan (pg/mL/cell) and cytokine (ng/mL/cell) concentration were evaluated using two-way ANOVA with Tukey's post hoc test. Comparison of gene expression values between two groups was performed using unpaired *t*-test or the non-parametric Mann-Whitney test and multiple comparisons were performed with Kruskal-Wallis and Dunn's post hoc tests. Values are presented as mean ± SEM of triplicate wells. In all analyses, the level of significance was considered as $P \leq 0.05$.

Table 1: Sequence of primers.

Gene name	Sequence
Beta-actin	5′-GGCACCCAGCACAATGAAG-3′
	5′-CCGATCCACACGGAGTACTTG-3′
HAS-1	5′-CAAGGCGCTCGGAGATTC-3′
	5′-CCAACCTTGTGTCCGAGTCA-3′
HAS-2	5′-CAGACAGGCTGAGGACGACTTTAT-3′
	5′-GGATACATAGAAACCTCTCACAATGC-3′
HAS-3	5′-GGCGATTCGGTGGACTACAT-3′
	5′-CGATGGTGCAGGCTGGAT-3′
HYAL-1	5′-GGTGAGCTGGGAAAATACAAGAA-3′
	5′-GCCCCAGTGTAGTGTCCATATACTC-3′
HYAL-2	5′-GGCGCAGCTGGTGTCATC-3′
	5′-CCGTGTCAGGTAATCTTTGAGGTA-3′
HYAL-3	5′-TGTGCAGTCCATTGGTGTGA-3′
	5′-AAGGTGTCCACCAGGTAGTCATG-3′
Collagen type I	5′-CCGCCGCTTCACCTACAGC-3′
	5′-TTTGTATTCAATCACTGTCTTGCC-3′
Collagen type II	5′-CCGAATAGCAGGTTCACGTACA-3′
	5′-CGATAACAGTCTTGCCCCACTT-3′
Aggrecan	5′-TTCAGTGGCCTACCAAGTGG-3′
	5′-AGCCTGGGTTACAGATTCCA-3′
Sox-9	5′-TGCTAGAAGATGAGGCTTCTGG-3′
	5′-GGCACTTTGTCCAGACCCA-3′

3. Results

3.1. The Number of BM-MSCs Is Decreased When Cocultured with OA-Chondrocytes. To determine effects of the cell coculture we counted the cell number of both BM-MSCs and OA-chondrocytes remaining at the end of the coculture and compared them to the corresponding cell number when cultured alone (Figures 1(a) and 1(b)). After three days in coculture with chondrocytes, BM-MSCs count decreased 28% compared to BM-MSCs cultured alone (7.6×10^4 versus 10.6×10^4). The difference increased as time in culture progressed and after six days BM-MSCs numbers were only 53% of the cells cultured alone (6.3×10^4 versus 13.5×10^4). We observed that the number of BM-MSCs decreased after 6 days in coculture with OA-chondrocytes in comparison to 3 days (7.6×10^4 versus 6.3×10^4), but no statistical difference was observed. The number of OA-chondrocytes cultured together with BM-MSCs, however, did not change significantly at both time points analyzed (Figure 1(b)). In spite of these changes, no significant variation in cell ratio was observed between BM-MSCs and OA-chondrocytes in coculture, after 3 days (75,920 BM-MSCs and 63,574 OA-chondrocytes, 1.2 : 1 cell ratio) and 6 days (65,000 BM-MSCs and 71,944 OA-chondrocytes, after 3 and 6 days, resp., i.e., 0.9 : 1 cell ratio).

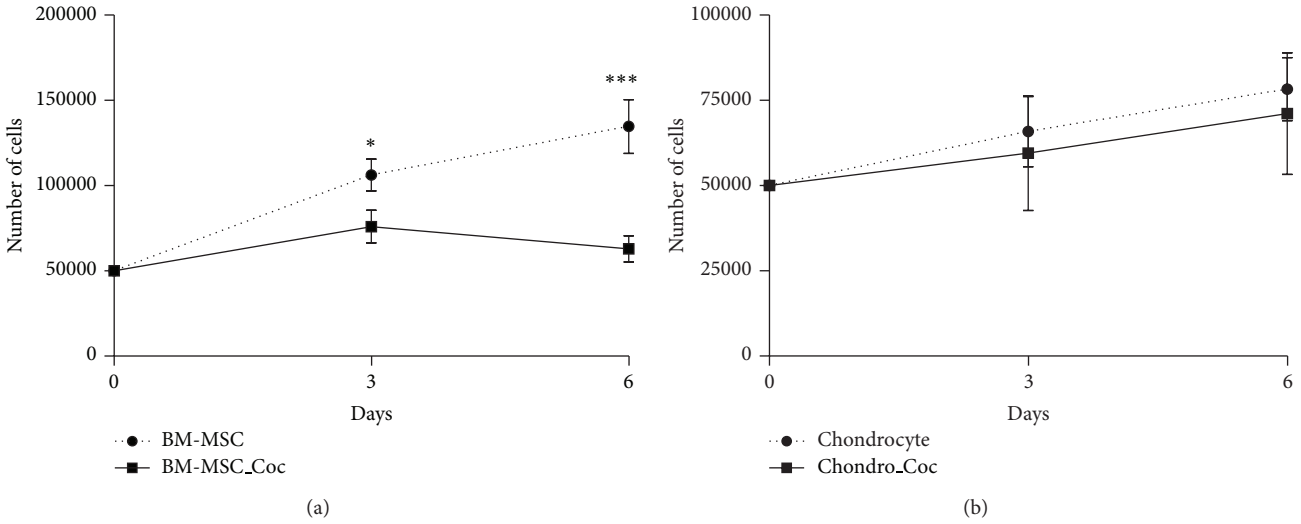
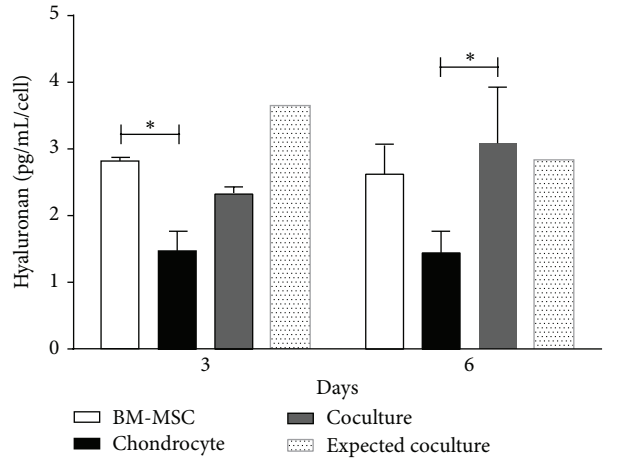


Figure 1: BM-MSCs decrease cell number after coculture with OA-chondrocytes. Number of cells cultivated for 3 and 6 days in monoculture ($n = 6$; bone marrow mesenchymal stem cells (BM-MSCs) and chondrocytes) or in coculture ($n = 6$; BM-MSC_Coc and Chondro_Coc). Error bars represent the SEM for the mean value. Statistical significance (two-way ANOVA) is set according to the number of asterisks, as follows: $^{*}P \leq 0.05$, $^{***}P \leq 0.0001$.



	Cell number					
BM-MSC	10.5×10^4	—	7.6×10^4	12.4×10^4	—	6.5×10^4
Chondrocyte	—	6.6×10^4	6.3×10^4	—	8.3×10^4	7.2×10^4

Figure 2: Coculture upregulates hyaluronan production. Hyaluronan concentration (pg/mL/cell) after 3 and 6 days in monoculture (BM-MSCs and chondrocytes) or in coculture. Bars represent the mean and SEM; bars with gray line (expected coculture) show "expected hyaluronan production in coculture," based on production of monoculture and cell number counted. Table shows the cell number in each group. The asterisk (∗) indicates a significant ($P \leq 0.05$) difference between cell culture groups based on a two-way ANOVA followed by Tukey's posttest.

3.2. BM-MSCs and OA-Chondrocyte Coculture Modulates Hyaluronan Production. To determine whether hyaluronan synthesis could be altered by coculture, we measured hyaluronan secreted by the cells in monoculture and when cocultured. Data on hyaluronan concentration obtained were normalized by cell number, in order to account for the greater number of cells in the coculture. To evaluate individual OA-chondrocyte and BM-MSC contribution in coculture, expected values were calculated based on the sum of values obtained from individual OA-chondrocyte and BM-MSC (monoculture) normalized by cell number of each cell type. We anticipated that the comparison between the expected and observed values could clarify whether the cross talk between OA-chondrocytes and BM-MSCs in coculture results in changes in hyaluronan production. Our results showed that both cells were able to synthesize hyaluronan, albeit BM-MSCs produced 2-fold more hyaluronan than OA-chondrocytes (2.80 pg/mL/cell versus 1.5 pg/mL/cell, resp.) after 3 days in monoculture (Figure 2).

Hyaluronan production per cell was increased in coculture when compared with OA-chondrocytes cultivated alone (Figure 2). On a per cell basis, after 6 days in our coculture system, hyaluronan present in the supernatant was 2.15-fold higher than that from OA-chondrocytes alone (3.09 pg/mL/cell versus 1.45 pg/mL/cell; Figure 2) but was similar to the levels detected in BM-MSCs monocultures (2.6 pg/mL/cell). Though, after 3 days, hyaluronan levels show intermediate values, the same is not true for values obtained after 6 days in coculture, clearly much higher. The expected values if hyaluronan production ratio was maintained after 6 days would be 2.7 pg/mL/cell (1.3 pg/mL/cell BM-MSC + 1.4 pg/mL/cell OA-chondrocyte) in contrast to the value of 3.09 pg/mL/cell observed.

Coculture is an important experiment when assessing cell:cell interactions. In our study cells were cocultured in a Transwell system that permitted harvesting of cells at the two defined time points and showed that though present proliferation rate of BM-MSCs was significantly decreased (cell number, Figure 2). On the other hand, products are

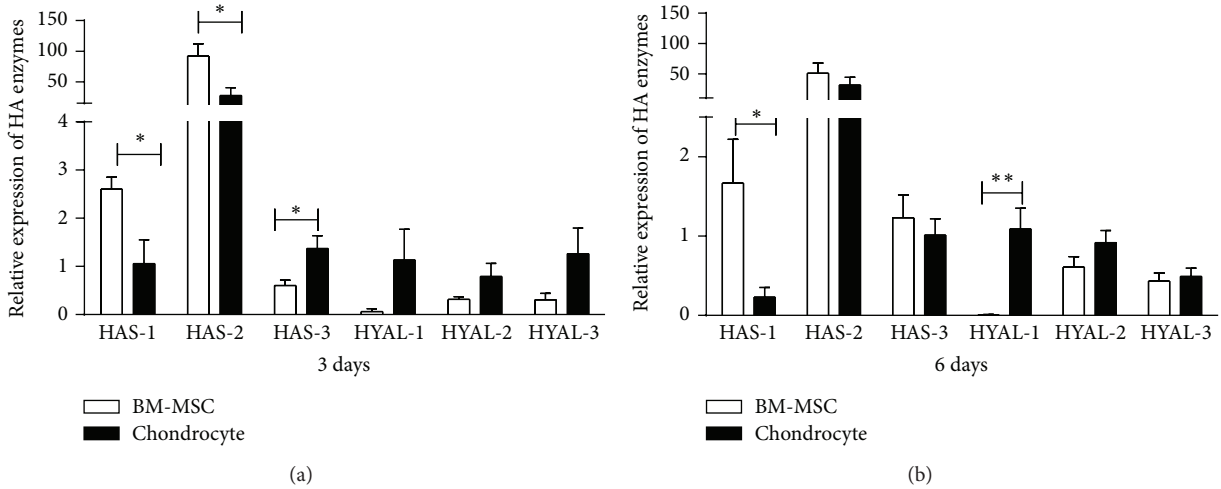


Figure 3: mRNA expression of hyaluronan enzymes by BM-MSCs and OA-chondrocytes. Relative mRNA expression of hyaluronan-related enzymes after 3 days (a) or 6 days (b) in monoculture. Bars represent the mean ± SEM of hyaluronan synthase- (HAS-) 1, HAS-2, and HAS-3 and hyaluronidase- (HYAL-) 2 and HYAL-3 mRNA expression. All fold changes were calculated relative to a calibrator sample. Statistical significance based on unpaired t-test was set according to the number of asterisks, as follows: $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

continuously secreted into the supernatant making this analysis more difficult. Alternatively, in BM-MSC and OA-chondrocyte cultured alone, cell number increased, but no change in hyaluronan production was observed during the time points. In coculture hyaluronan concentration increased during time points, without changing the total cell number.

3.3. Hyaluronan-Related Enzymes Are Differentially Expressed in OA-Chondrocytes and BM-MSCs. To further clarify the contribution of each cell type to hyaluronan production, we first measured hyaluronan synthase- (HAS-) 1, HAS-2, and HAS-3 and hyaluronidases- (HYAL-) 1, HYAL-2, and HYAL-3 mRNA expression in monocultures as mean fold change relative to a calibration sample. After 3 and 6 days in culture a distinct expression pattern of HAS was observed. BM-MSCs presented significantly greater HAS-1 expression than OA-chondrocytes (Figures 3(a) and 3(b)); in the latter HAS-1 was practically absent after 6 days in culture. HAS-2 also varied with higher relative values exhibited by BM-MSCs. HAS-3 gene expression was low in both cells and at both time points. On the other hand, mRNA expression of the three hyaluronidases was low at both time points (Figures 3(a) and 3(b)).

After 3 days in coculture relative HAS-1 mRNA expression by BM-MSCs was further increased compared to BM-MSCs cultured individually (3.67-fold, Figure 4(a)). After 6 days we observed a trend towards increase of HAS-1 mRNA expression by BM-MSC (Figure 4(b)). In contrast, we observed a downregulation (~25-fold) of HYAL-1 mRNA expression in BM-MSC after interaction with OA-chondrocyte at both time points (after 3 and 6 days; Figures 4(c) and 4(d)). HYAL-2 mRNA expression was also reduced after 6 days (1.5-fold) (Figure 4(d)). The expression of other enzymes was unaltered in BM-MSCs and no change in the expression of any of the enzymes was detected in the cocultured OA-chondrocytes in comparison with OA-chondrocytes in monoculture (Figures 5(a)–5(d)).

3.4. Gene Expression of Extracellular Matrix Components. Alteration in genes related to extracellular matrix components may reflect cartilage regeneration and differentiation status. Thus, we chose to evaluate whether coculture affects the mRNA expression of type I and type II collagen, Sox-9, and aggrecan. Our findings showed that OA-chondrocytes maintained the expression of chondrogenic markers throughout the experiment. No significant difference in expression of extracellular matrix genes (type I and II collagen, aggrecan) and Sox-9 was observed after 3 or 6 days of coculture system (Supplementary Figures 1(a) and 1(b) in Supplementary Material available online at http://dx.doi.org/10.1155/2015/640218).

3.5. Coculture Alters Cytokine Production. To evaluate the effects on the inflammatory microenvironment, we measured six cytokines (IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70) in the culture supernatants of OA-chondrocytes and BM-MSCs cocultures. Only IL-6 and IL-8 were present in detectable levels and there was no evidence of production of the remaining cytokines investigated. Similarly to hyaluronan synthesis, we normalized cytokine production by the cell number measured at 3 and 6 days.

As expected, OA-chondrocytes produced 8-fold greater amounts of IL-6 than BM-MSC after 3 days (113 ng/mL/cell versus 14 ng/mL/cell), a difference maintained at 9-fold after 6 days (238 ng/mL/cell versus 25 ng/mL/cell; Figure 6(a)). Similarly, OA-chondrocytes produced more IL-8 than BM-MSCs, a 14-fold increase after 3 days (130 ng/mL/cell versus 9 ng/mL/cell) and a 21-fold increase after 6 days (107 ng/mL/cell versus 5 ng/mL/cell; Figure 6(b)). Interestingly, as a result of coculture IL-6 secretion per cell was

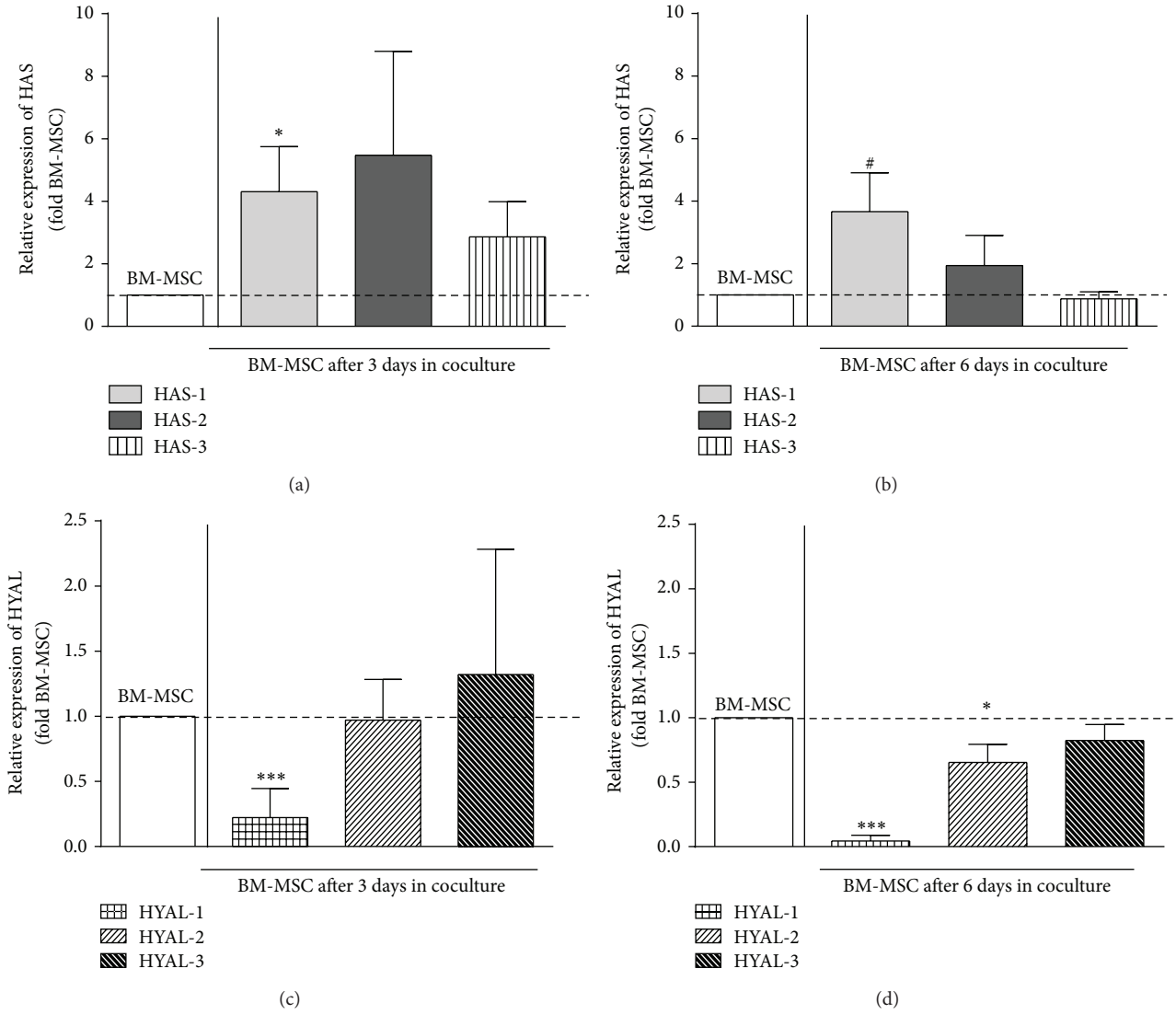


Figure 4: Hyaluronan enzyme mRNA expression in BM-MSC after coculture. mRNA expression of hyaluronan synthase- (HAS-) 1, HAS-2, and HAS-3 (a-b) and hyaluronidase- (HYAL-) 1, HYAL-2, and HYAL-3 (c-d) after coculture relative to time-matched and cell-matched controls. Expression of HAS in cocultivated BM-MSC relative to BM-MSC monoculture after 3 days (a) and 6 days ($n = 5$) (b); expression of hyaluronidases in cocultivated BM-MSC relative to BM-MSC monoculture after 3 days (c) and 6 days (d) ($n = 5$). Bars represent the mean fold change value ± SEM relative to BM-MSC in monoculture of hyaluronan enzymes. Statistical significance based on unpaired t-test was set according to the number of asterisks, as follows: $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

of lower levels when compared to chondrocytes in monoculture (55 ng/mL/cell versus 113 ng/mL/cell) and of greater levels when compared to BM-MSCs (55 ng/mL/cell versus 14 ng/mL/cell). This pattern was also observed after 6 days (130 ng/mL/cell versus 238 ng/mL/cell, coculture versus OA-chondrocyte, and 25 ng/mL/cell BM-MSC, resp., Figure 6(a)). In contrast to the increase in hyaluronan observed, the secretion of IL-6 by OA-chondrocytes was clearly downregulated. The expected values if secretion levels were maintained would be 249 ng/mL/cell (12 ng/mL/BM-MSC + 237 ng/mL/OA-chondrocyte = 249 ng/mL/cell) but were only 130 ng/mL/cell. The same occurred with IL-8 where expected values would be 109 ng/mL/cell (i.e., 2.3 ng/mL/BM-MSC + 107 ng/mL/OA-chondrocyte) but reached only 30 ng/mL/cell, indicating a trend for lower production.

IL-8 measured after coculture was also lower than when OA-chondrocytes were cultured individually but did not reach statistical difference after both 3 (130 ng/mL/cell versus 51 ng/mL/cell) and 6 days (107 ng/mL/cell versus 30 ng/mL/cell, Figure 6(b)). Although the IL-8 concentration in coculture observed (30 ng/mL/cell) was different from the expected (109 ng/mL/cell, i.e., 2.3 ng/mL/BM-MSC + 107 ng/mL/OA-chondrocyte), we did not reach statistical difference between coculture and OA-chondrocytes.

4. Discussion

In the present study we sought to establish the impact of autologous BM-MSC on hyaluronan production and their effects on the secretion profile of chondrocytes from patients

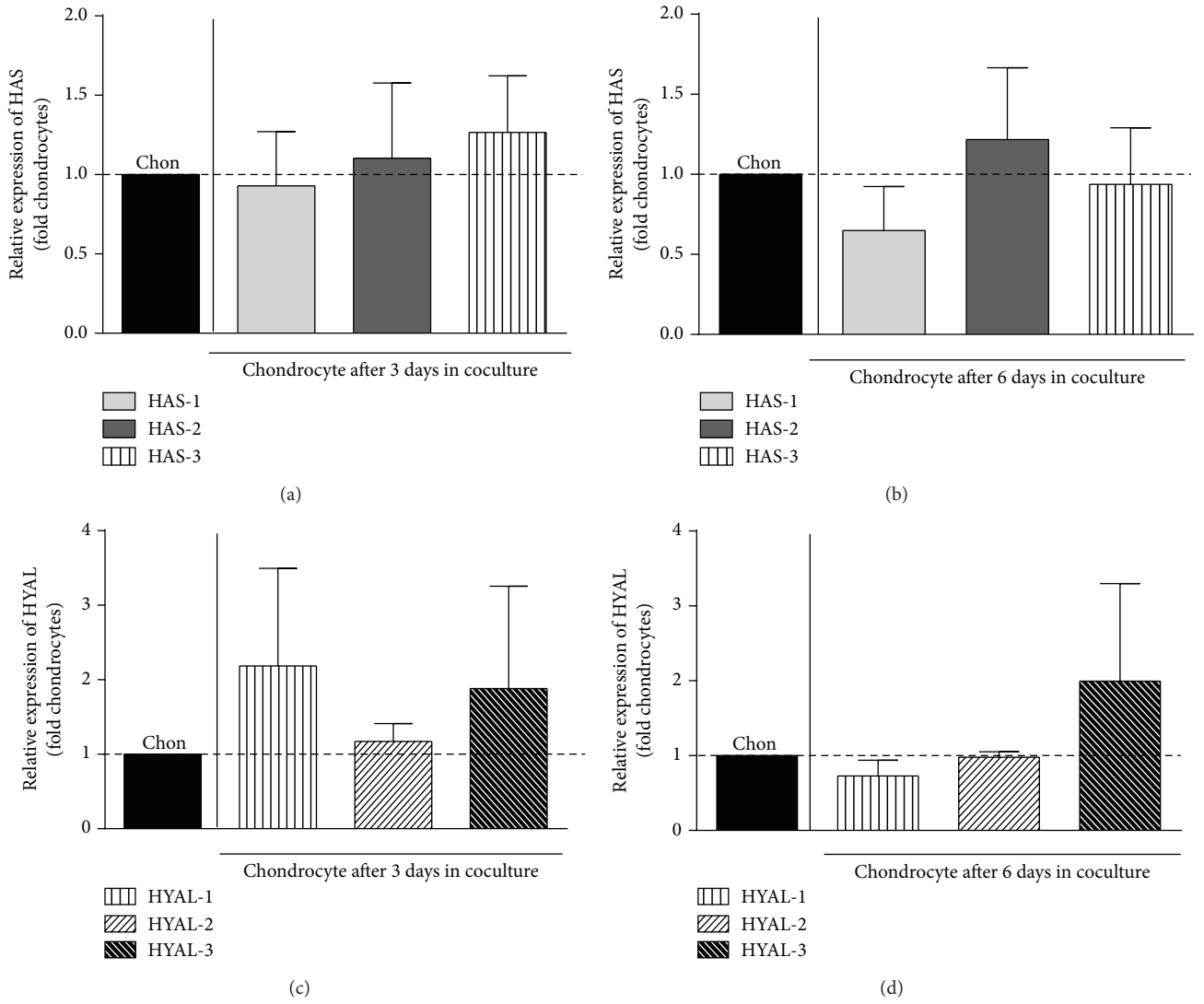
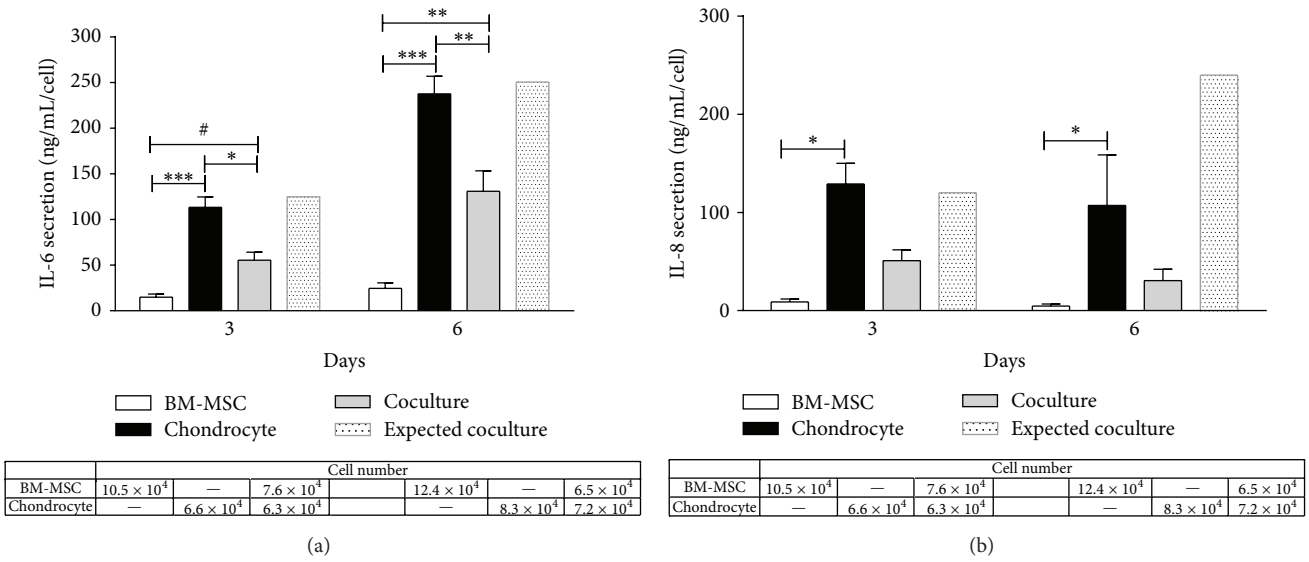


Figure 5: Hyaluronan enzyme mRNA expression in OA-chondrocytes after coculture. mRNA expression of hyaluronan synthase- (HAS-) 1, HAS-2, and HAS-3 (a-b) and hyaluronidase- (HYAL-) 1, HYAL-2, and HYAL-3 (c-d) after coculture relative to time-matched and cell-matched controls. Expression of HAS in cocultivated chondrocytes (Chon) relative to Chon monoculture after 3 days (a) and 6 days (b) ($n = 5$); expression of hyaluronidases in cocultivated chondrocyte relative to Chon monoculture after 3 days (c) and 6 days (d) ($n = 5$). Bars represent the mean fold change value ± SEM relative to chondrocyte in monoculture of hyaluronan enzymes. Statistical significance based on unpaired t-test was set according to the number of asterisks, as follows: $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

with OA. Our coculture experiments suggest that an interaction occurs between BM-MSC and OA-chondrocytes in a Transwell system, which favors hyaluronan production. Moreover, we were able to show that BM-MSCs alone produce high amounts of hyaluronan and exhibit abundant HAS-1 mRNA expression.

Hyaluronan is a key component of the cartilage matrix and is used widely as an anti-inflammatory and antinociceptive agent in the treatment of OA, improving joint lubrication and shock absorbance [24, 25]. Intra-articular hyaluronan injection has been employed in the management of patients with OA. The anti-inflammatory, anabolic, and chondroprotective action of hyaluronan has been increasingly evidenced, suggesting that hyaluronan helps to reduce pain and improve cartilage function [26]. Therefore, insights into mechanisms that can change hyaluronan levels in OA are relevant.

Several studies have investigated the effects of MSCs on chondrocytes [18, 27–29], but few have provided data to show that MSCs, and not only chondrocytes, might also be affected by the cell:cell interactions. Our study shows that coculture of BM-MSCs with OA-chondrocytes led to a decrease in BM-MSCs cell numbers which can be explained by reduced cell proliferation or cell death. However, cell death was not directly measured in the present study. These low BM-MSCs numbers after 3 or 6 days in coculture suggest that OA-chondrocytes are capable of altering BM-MSCs behavior. Similar results have been shown in studies using coculture of BM-MSCs with chondrocyte pellets from



	Cell number						
BM-MSC	10.5×10^4	—	7.6×10^4		12.4×10^4	—	6.5×10^4
Chondrocyte	—	6.6×10^4	6.3×10^4		—	8.3×10^4	7.2×10^4

(a)

	Cell number						
BM-MSC	10.5×10^4	—	7.6×10^4		12.4×10^4	—	6.5×10^4
Chondrocyte	—	6.6×10^4	6.3×10^4		—	8.3×10^4	7.2×10^4

(b)

Figure 6: OA-chondrocytes produce higher IL-6 and IL-8 levels than BM-MSCs and cocultured cells and decrease these cytokines. Interleukin (IL) production for 3 and 6 days in monoculture ($n = 5$; BM-MSCs and chondrocytes) or in coculture ($n = 5$). IL-6 concentration (ng/mL/cell) (a). IL-8 concentration (ng/mL/cell) (b). Overall, bars represent the mean value with the SEM; bars with gray line (expected coculture) show expected ILs synthesis in coculture, based on production of monoculture and cell number counted. Table shows cell number in each group. Statistical significance based on a two-way ANOVA with Tukey's posttest was set according to the number of asterisks, as follows: $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, and $^{***}P \leq 0.0001$. Tendency of statistical differences between cell culture groups was identified by #.

different sources, during 3- to 4-week periods of culture, in which BM-MSCs numbers decreased progressively and differentiated into chondrocytes [18, 30]. Thus, it is possible that in the present short-term cultures the trend towards differentiation into chondrocytes with a slowdown of the proliferation rate was already present and that an extended culture time would eventually favor chondrogenic marker alterations. In fact, in a previous study employing coculture with direct contact between MSCs and OA-chondrocytes, the increase in chondrogenic markers was observed later, beginning only after day 7 [31]. Nevertheless, our findings show that a short culture period is necessary and sufficient to change hyaluronan production in this microenvironment.

Hyaluronan production by MSCs has been recently described [32], but we have shown that an at least twice greater hyaluronan production occurs by BM-MSCs from the same patient than by OA-chondrocytes themselves. In addition, when BM-MSCs were cultured together with OA-chondrocytes, the pattern of increased production was still maintained although at lower rates than when BM-MSCs were cultured alone. Reduction in BM-MSC cell number concomitant with no change in OA-chondrocyte number most probably affected hyaluronan production. Given that the expected and observed values of hyaluronan showed significant differences, it can be concluded that BM-MSCs contribute to hyaluronan production in our coculture system.

Thus, the present study provides evidence for a role of BM-MSCs in hyaluronan synthesis in OA. Our values for hyaluronan production were similar to the short-term (1 day) BM-MSCs monolayer cultures (range between 2.25 and 49.74 pg/mL/cell [32]) even though we cultured the cells for longer periods (6 days).

Hyaluronan enzymes HAS-1 and HAS-2 have previously been shown to be downregulated in chondrocytes collected from OA cartilage when compared to the primary chondrocytes or cartilage [33, 34]. We measured mRNA expression levels of hyaluronan synthases to clarify which and to what extent BM-MSCs and OA-chondrocytes contribute to hyaluronan levels produced. Our data show that HAS-1, but not other isoforms, is increased in BM-MSCs when compared to OA-chondrocytes, suggesting that HAS-1 may have an important role in the hyaluronan production in our system. HAS-1 is the preponderant hyaluronan synthase present in native cartilage, which synthesized the high molecular weight hyaluronan which is found in normal cartilage [33]. The greater HAS-1 expression in BM-MSCs after coculture with OA-chondrocytes indicates that BM-MSCs might be capable of upregulating hyaluronan production in OA cartilage, though. The cocultured OA-chondrocytes exhibited unchanged expression of HASs after 3 or 6 days in culture. It is possible that 6 days is not enough time to induce this change. It is also important to note that a 3D culture could eventually induce more rapid changes as a result of the BM-MSCs secreted factors.

Hyaluronan levels in the extracellular matrix and, ultimately, the regenerative potential in cartilage are determined not only by hyaluronan synthesis and but also by its degradation. Therefore, expression of enzymes involved in hyaluronan degradation was analyzed in OA-chondrocytes and in BM-MSCs. Among these enzymes, HYAL-1 has been described as an enzyme critical for cartilage development [8]. HYAL-1 mRNA was detected at higher levels in OA-chondrocytes than in BM-MSCs cultured alone. To our knowledge, the present study is the first to show mRNA

expression profile of HYALs in BM-MSCs in comparison with OA-chondrocytes. Our results show that HYAL-1 and HYAL-2 mRNA expression are downregulated in BM-MSCs after coculture with OA-chondrocytes, in concordance with the higher amounts of hyaluronan found in this system. Because HYAL-1 and HYAL-2 hydrolyze hyaluronan fragments of different sizes and have been suggested as the most abundant hyaluronan-degrading enzymes, these enzymes may be working together to degrade hyaluronan in OA [10, 11]. The expression of other enzymes did not change in BM-MSCs and OA-chondrocytes after coculture. Thus, our data suggest that OA-chondrocytes modulate BM-MSCs by increasing HAS-1 and inhibiting HYAL-1 and HYAL-2 expression in order to synthesize higher molecular weight hyaluronan and, consequently, improve the local microenvironment.

The beneficial effect of hyaluronan on cartilage regeneration was demonstrated in an *in vivo* study using hyaluronan hydrogel combined with MSC [35]. The hyaluronan production by BM-MSCs might also have a direct anti-inflammatory role. Hyaluronan injection in the knees of OA patients has been associated with decreased IL-6, but not with IL-8 levels in the synovial fluid, which correlated with clinical improvement [36]. Another study suggested that the presence of hyaluronan reduces TNF-α and IL-6 concentration in coculture of OA-cartilage explants with synoviocytes [37].

Osteoarthritic cartilage is typically characterized by the presence of cytokines associated with inflammation, such as interleukin-1 beta (IL-1β), IL-10, IL-6, and TNF-α, besides proteolytic molecule MMPs. These cytokines are secreted by chondrocytes and contribute to OA development [13, 38]. Our results show that OA-chondrocytes in culture maintain secretion of high levels of inflammatory molecules even in the presence of interfering factors such as fetal calf serum. These OA-chondrocytes produced large amounts of IL-6 and IL-8 even though kept for long periods in culture, suggesting that they preserve an "inflammatory memory." In contrast, BM-MSCs obtained from the same individual showed low levels of IL-6 and IL-8 production. In fact, coculture of OA-chondrocytes with the paired BM-MSCs reduced IL-6 secretion on a "per cell" basis. These observations are consistent with a report showing an anti-inflammatory effect of adipose-derived allogeneic MSC on OA-chondrocytes with a decrease of IL-6 and IL-8 production [27]. A differential production of IL-8 was, however, not detected in our analyses.

The beneficial hyaluronan production and anti-inflammatory role of BM-MSCs indicate that the cross talk with OA-chondrocytes may stimulate synthesis of other soluble molecules creating a more propitious environment for cartilage regeneration. Experimental models that permit cell contact (using OA cartilage explants) suggested that the microenvironment of OA cartilage does affect the chondrogenic potential of BM-MSCs [39].

The fact that in our study coculture was established without cell:cell contact opens new avenues of cell therapy using even allogeneic MSCs, which would induce short-term changes, by adding hyaluronan and blockading IL-6 and IL-8, to induce a more regenerative and less inflammatory microenvironment in the affected OA cartilage.

5. Conclusion

BM-MSCs produce hyaluronan and modulate this production in response to cross talk with OA-chondrocytes.

In conclusion, our data support the hypothesis that BM-MSCs produce hyaluronan in response to OA-chondrocytes, increasing mRNA expression of HAS-1 associated with HYAL-1 downregulation and hyaluronan synthesis. The interaction promoted also an overall lower IL-6 production. Taken together, these results indicate that BM-MSCs *per se* can be a potential tool for OA regenerative therapy. Our study offers insights into the mechanisms whereby treatment with BM-MSCs would exert beneficial effects on the diseased cartilage as a therapeutic strategy to increase hyaluronan production and decrease inflammation locally. More importantly, our data point to a strategic role of MSCs in differentiating into more active, specialized cells and not only in remodeling chondrocytes. However, more basic and preclinical studies that consider MSC as an alternative OA treatment are needed.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

All authors read and approved the final paper. Eliane Antonioli, Mario Ferretti, and Anna Carla Goldberg contributed to the conception and design; Eliane Antonioli, Mario Ferretti, and Moisés Cohen contributed to collection and assembly of data; Eliane Antonioli and Helena B. Nader performed experiments and data acquisition; Eliane Antonioli, Carla A. Piccinato, and Mario Ferretti contributed to data analysis and interpretation; Eliane Antonioli, Carla A. Piccinato, Mario Ferretti, and Anna Carla Goldberg contributed to writing; Helena B. Nader and Anna Carla Goldberg contributed to editing; Mario Ferretti contributed to financial and administrative support.

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Review Article

Low Reactive Level Laser Therapy for Mesenchymal Stromal Cells Therapies

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Low reactive level laser therapy (LLLT) is mainly focused on the activation of intracellular or extracellular chromophore and the initiation of cellular signaling by using low power lasers. Over the past forty years, it was realized that the laser therapy had the potential to improve wound healing and reduce pain and inflammation. In recent years, the term LLLT has become widely recognized in the field of regenerative medicine. In this review, we will describe the mechanisms of action of LLLT at a cellular level and introduce the application to mesenchymal stem cells and mesenchymal stromal cells (MSCs) therapies. Finally, our recent research results that LLLT enhanced the MSCs differentiation to osteoblast will also be described.

1. Introduction

Mesenchymal stromal cells (MSCs) are the promising source for the regenerative medicine and repair of various tissues in the treatment of a range of diseases. The differentiation of these cells to different lineages is dictated by the local extracellular matrix (ECM) as well as spatial and temporal cues, including growth factors and cell-cell interactions. In bone formation, mechanotransduction and physical cues, such as shear stress and fluid flow [1], also influence the differentiation of MSCs. However, the fundamental questions that how to control the differentiation of MSCs to matured cells must be answered. In particular, a better understanding of how specific factor may alter the fate of differentiation of MSCs is needed. Their rapid and selective differentiation should provide the potential of new therapeutic approaches for the restoration of damaged or diseased tissue. We have reported that the laser irradiation to MSCs influences cell differentiation and possible mechanisms of cells differentiation by laser were proposed [2–4]. In this review, we will describe the mechanisms of action of laser irradiation and introduce the application to MSCs therapies including our research results.

A laser (light amplification by stimulated emission of radiation) is a device that generates electromagnetic radiation that is relatively uniform in wavelength, phase, and polarization. This technology was originally described by Maiman in 1960 in the form of a ruby laser [5]. The properties of lasers have allowed for numerous medical applications, including their use in surgery, activation of photodynamic agents, and various ablative therapies in cosmetics, all of which are based on heat generated by the laser beam, in some cases leading to tissue destruction [6–13]. Low reactive level laser therapy (LLLT) is a form of medical treatment in which human tissue is irradiated with a low-powered laser (on the order of several hundred milliwatts) to induce therapeutic changes. In an attempt to explore the carcinogenic potential of laser light, Mester et al. in 1967 applied a low-powered ruby laser with a 694 nm wavelength to the shaved dorsal skin of mice [14]. Contrary to their expectations, the laser irradiation did not cause cancer but instead improved hair growth. As the first study to document the biological effect of lasers, their findings became a springboard for subsequent LLLT research. Although light-based therapies had been used for a long time and ultraviolet therapy has a history longer than a century [15], the work of Mester et al. was significant in demonstrating the effects of laser light, which has the unique characteristics of monochromaticity and coherence. Following subsequent experiments, Mester and colleagues reported in 1971 that low power laser rays accelerated wound healing [16]. Since those

early days, numerous *in vitro* and *in vivo* studies of LLLT in the context of regenerative medicine have demonstrated a wide variety of therapeutic effects including improvements in wound healing, collagen synthesis, cell proliferation, fracture repair, and local blood circulation, as well as suppression of inflammation and pain. According to da Silva et al. [17], the types of laser most frequently used for wound healing and tissue repair are helium-neon (He-Ne) lasers and diode lasers, including gallium-aluminum-arsenic (Ga-Al-As), arsenic-gallium (As-Ga), and indium-gallium-aluminum-phosphide (In-Ga-Al-P) lasers.

A large number of literatures and review articles [18–20] have shown that LLLT accelerates wound healing, and we present some typical results here. Irradiation of cultured human keratinocytes with a 632 nm helium-neon laser elevated the interleukin-1α and interleukin-8 mRNA levels, promoted keratinocyte migration and proliferation, and accelerated wound repair [21]. In addition, *in vitro* studies of laser-irradiated cells revealed elevated levels of vascular endothelial growth factor (VEGF) [22] and transforming growth factor β (TGF β) expression [23]. These findings illustrate the laser-enhanced expression of many cytokines and growth factors in keratinocytes and fibroblasts, the key cellular mediators of the wound-healing process. In addition, after mice with lipopolysaccharide-induced peritonitis were irradiated with a 904 nm gallium arsenide (Ga-As) laser, inflammatory cell migration was inhibited [24]. In a rat model of carrageenan-induced pleuritis, a 660 nm In-Ga-Al-P laser suppressed the production of inflammatory cytokines and the migration of inflammatory cells [25]. A group of researchers led by Albertini are actively pursuing research on LLLT's anti-inflammatory effects [26–46]. In the field of regenerative medicine, LLLT accelerates osteoblast proliferation, bone formation [47], and bone repair [48]. Various groups have suggested the involvement of insulin-like growth factor-1 (IGF-1) [49], mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) [50], and bone morphogenetic protein (BMP)/Smad signaling cascades [51]. In addition, LLLT confers physiological effects to regeneration of damaged neurons [52–55], articular cartilage [56], and muscle tissue [57–59]. To date, several mechanisms of biological action have been proposed, although none have been clearly established. These include augmentation of cellular ATP levels [60–62]; manipulation of inducible nitric oxide synthase (iNOS) activity [63–67]; suppression of inflammatory cytokines such as tumor necrosis factor- (TNF-) α [61, 68–70], interleukin- (IL-) 1β [27, 70, 71], IL-6 [25, 70, 72–74], and IL-8 [25, 70, 72, 75]; upregulation of growth factors such as PDGF, IGF-1, NGF, and FGF-2 [71, 76–78]; alteration of mitochondrial membrane potential [79–82] due to chromophores found in the mitochondrial respiratory chain [83–85]; stimulation of protein kinase C (PKC) activation [86]; manipulation of nuclear factor- (NF-) κB activation [28]; induction of reactive oxygen species (ROS) [87, 88]; modification of extracellular matrix components [89]; inhibition of apoptosis [79]; stimulation of mast cell degranulation [90]; and upregulation of heat shock proteins [91].

In the following paragraphs, we will discuss the cellular effects of LLLT that underlie its biological actions. Through our research, we have discovered (i) the presence of intracellular photoreceptors and physiological changes resulting from photoreception, (ii) postirradiation modifications in cellular signal transduction cascades, and (iii) postirradiation alterations in gene expression. These various effects do not occur in an isolated manner. Here, we will focus on how these effects interact with each other to induce modifications in cellular functions. We will also summarize typical results of the LLLT application to MSCs therapies.

2. Laser-Induced Cellular Responses

In order to elucidate the biological mechanisms underlying effects of low power lasers documented in experimental and clinical studies, one must consider the cellular responses to laser irradiation. The photons must be absorbed by electronic absorption bands belonging to some molecular chromophores or photoreceptors [92]. A chromophore or photoreceptor is a molecule (or part of a molecule) where the energy difference between electrons in two different molecular orbitals falls within the energy possessed by photons in the visible spectrum. In this section, we describe the intracellular photoreceptors and the cellular responses to laser light. One of the most distinctive features of LLLT relative to other modalities is that the effects are mediated not through induction of thermal effects but rather through a process called "photobiostimulation."

2.1. Intracellular Photoreceptor. In photobiology, photoreception refers to the intracellular process whereby wavelength-specific photoreceptors absorb photon energy [92]. Photoreceptors are biomolecules that are capable of absorbing photoenergy, either intrinsically or via a molecular component. The mitochondrial respiratory chain includes multiple photoreceptors, as described below.

2.1.1. Cytochrome c Oxidase. The enzyme cytochrome *c* oxidase receives electrons from respiratory-chain substrates via the cytochrome pathway and transfers them to oxygen molecules. Cytochrome *c* oxidase has been proposed as the endogenous photoreceptor in the visible to near-infrared region (above 600 nm) [93]. Scientists have conducted extensive research on the photobiomodulation by cytochrome *c* oxidase, particularly in neuronal cells. In a study of neurons functionally inactivated by tetrodotoxin, a voltage-dependent sodium channel blocker [94], near-infrared irradiation restored the activity of intoxicated cytochrome *c* oxidase by altering its redox state. In another study, laser irradiation of mitochondria increased cytochrome *c* oxidase activity, polarographically measured levels of oxygen uptake, and subsequent ATP production [95]. Many other *in vitro* and *in vivo* studies of laser-induced cell growth have reported changes in cytochrome *c* oxidase activity and ATP production following irradiation [81, 96–103].

2.1.2. Porphyrin. Porphyrins are a group of macrocyclic organic compounds that contain four pyrrole subunits joined by methine bridges. These mostly green- or red-colored compounds have specific absorption spectra and emit red fluorescence. Naturally occurring porphyrins, including those found in the human body, often form complexes with an iron or magnesium ion coordinated to the four pyrrole nitrogen atoms. For example, iron protoporphyrin IX (PPIX) complexes (i.e., heme *b*) form the prosthetic groups of hemoglobin, catalase, and peroxidase. Mitochondrial cytochromes also contain iron-porphyrin groups (nonheme *b*). The PPIX absorption spectrum has five major peaks in the range of 400 to 650 nm, with peak height decreasing as the absorption wavelength increases. The excited triplet state of PPIX, formed by absorption of laser photons, generates ROS by transferring energy to ground-state oxygen atoms. A mode of photodynamic therapy (PDT) that exploits this feature has been developed for anticancer treatment. In this technique, patients are administered PPIX or its precursor, 5-aminolevulinic acid (ALA), and ROS are generated with local laser irradiation to kill malignant cells or epithelial cells of vascular neoplasms [104].

2.1.3. Flavoproteins (Flavin Proteins). Flavoproteins are a group of protein complexes containing a riboflavin prosthetic group (e.g., flavin adenine dinucleotide [FAD] or flavin mononucleotide [FMN]). Most flavoproteins function as flavin enzymes, which use iron, molybdenum, copper, manganese, and other heavy metal ions as cofactors. These proteins have major absorption peaks in the range of 350 to 500 nm. Flavoproteins mediate a wide array of biological processes, such as bioluminescence, quenching of oxidative stress-induced radicals, DNA repair, and apoptosis [105]. Some researchers, including the present author, have reported the roles of flavoproteins as intracellular photoacceptors [2, 3, 106].

2.1.4. Other Groups of Photoreceptors. In addition to the three major groups of photoreceptors explained above, there are other types of photoreceptors, including rhodopsin, bilirubin, melanin, pterin, vitamin B6, vitamin K, nicotinamide adenine dinucleotide (phosphate) hydrogen [NAD(P)H], urocanic acid, and tryptophan.

2.2. Laser-Induced Changes in Signaling Cascades. It is clear that signal transduction pathways regulate cells in order to transduce the signal from the cellular photoreceptors that absorb photon energy to the biochemical machinery that controls gene transcription. Many researchers believe that the photon energy captured by intracellular receptors leads to alterations in gene and protein expression via a series of processes that modify signaling cascades. However, little is known regarding how light-stimulated receptors transduce their signals to the nucleus, or how these signals mediate the expression of particular genes. We have studied the mechanisms underlying the promotion and suppression of stem cell differentiation, with a focus on FAD-containing cryptochromes as cellular photoreceptors [2, 3]. Our research suggested that light-activated cryptochromes migrate into the nucleus, where they regulate the expression of proteins located downstream of the E-boxsequence. As a matter of course, cell functions are regulated by an array of other factors, including ROS. Therefore, we will now describe the biochemical changes LLLT induces beyond the photoreceptor absorption of light energy, as reported in the literature.

2.2.1. Redox Pathways. Several oxygen and nitrogen radicals have been proposed to transduce mitochondrial signals to the nucleus. Those species react with NAD, NADH, NADP, NADPH, glutathione, glutathione sulfide, thioredoxin, and thioredoxin sulfide [107]. The cell contains several endogenous sensors for these species (typically, superoxide dismutase [SOD]) [108]. Upon detection of ROS, the cell activates self-defense pathways by altering its gene expression patterns [109]. If these self-defense mechanisms fail, the cell will undergo apoptosis. The levels of ROS strictly determine the expression of proteins regulating cell proliferation, suggesting that oxygen radicals act as second messengers [110, 111]. ROS are considered to play key roles in the control of cellular functions [112]. Low power laser beams with wavelengths around 630 nm generate oxygen radicals in exposed cells [113, 114]. We have also discovered significant increases in the levels of oxygen radicals in cells exposed to laser light (wavelength: 405 nm) [87]. Although the specific mechanism remains unknown, laser-induced intracellular generation of ROS probably involves energy transfer from PPIX and other photoreceptors present in the cell. In addition, several groups have described cellular functions mediated by nitric oxide (NO), which is upregulated by laser irradiation, as well as by inducible nitric oxide synthase (iNOS) [65, 67, 114–116]. The mechanism of laser-induced control of cellular functions is believed to hinge on the regulation of photoreceptor activity and the intracellular levels of ROS.

2.2.2. Transcription Factors. Several researchers have reported that the aforementioned redox pathways trigger changes in the expression of many transcription factors. Here, we briefly describe one of the best-characterized transcription factors in the LLLT field, NF-κB [117, 118]. Published articles on other transcription factors mediating a multitude of cell functions have made it clear that their expression levels are also modified upon exposure to laser irradiation. As a transcription factor, NF-κB can simultaneously induce the expression of IL-1, IL-2, IL-6, IL-8, IL-12, TNF-α, and other proinflammatory cytokines. It also controls the expression of apoptosis-related proteins, which play a critical role in tumor cell growth and immortalization. Several studies have shown that the aforementioned redox pathways trigger increases in NF-κB levels [117, 118]. This mechanism is considered to account, at least in part, for the observation that low power laser irradiation induces the expression of various cytokines. Rizzi et al. have showed that histological abnormalities with increase in collagen concentration and oxidative stress were observed after trauma. The associated reduction of inducible nitric oxide synthase overexpression and collagen production suggest

that the NF-κB pathway is a signaling route involved in the pathogenesis of muscle trauma [118]. The hypoxia-inducible factor (HIF-1) is also a ubiquitous transcription factor involved in the control of cell and tissue responses to hypoxia, specifically in angiogenesis, hematopoiesis, and anaerobic energy metabolism. There are over 70 genes which have been established as direct targets by identification of critical HIF-1 binding sites [119]. In addition, the activator protein- (AP-) 1 is involved in cellular proliferation, transformation, and death [120]. AP-1 is not a single protein but a complex array of heterodimers composed of proteins that belong to the Jun, Fos, and ATF subfamilies, which recognize specific nuclear target sequences. Different dimeric combinations can stimulate a variety of gene expression patterns. AP-1 can be activated by growth factors, cytokines, hypoxia, ionizing, and UV radiation [121, 122].

2.2.3. Circadian Rhythm. The circadian rhythm, a roughly 24-hour cycle of cellular events, was acquired during the early stages of evolution and is ubiquitous from unicellular organisms to mammals. Several mammalian clock genes work together to establish a stable oscillation of approximately 24 hours. Circadian clock proteins, such as brain-muscle Arnt-like protein 2 (BMAL2), clock, cryptochrome (CRY), and period (PER), set the pace of the clock in almost all cell types (e.g., the timing of cell division and other cellular activities). CRY, a blue-light receptor in higher plants and Drosophilidae [123], utilizes as its chromophore the FAD coenzyme, which undergoes blue-light excitation. This observation led to the idea that light-excited FAD transfers electrons to a certain substrate. However, the validity of this theory has not been tested. Bone metabolism (remodeling) is a continuous homeostatic process involving resorption of existing bone by osteoclasts and formation of new bone by osteoblasts. Fu et al. showed that circadian rhythms mediate bone formation [124], and Kawasaki et al. reported that the E-box motif, a circadian regulatory sequence, is involved in the osteoblast expression of bone morphogenetic protein- (BMP-) 4 [125]; these findings indicate that CRY proteins regulate various homeostatic and physiological events via E-box elements. We conducted research on the effects of lasers on endocellular distribution and expression of CRY using laser beams (wavelength: 405 nm), which correspond to the absorption band of the CRY coenzyme FAD [2]. We will describe the results below.

3. LLLT for MSCs Therapies

Since LLLT has been scientifically proven as a beneficial therapeutic modality for numerous diseases and diseased conditions, it was applied to enhance MSCs proliferation and differentiation. The recent 3-year reports regarding LLLT application to increase MSCs proliferative and differentiation potential were summarized in Table 1 [126–135, 138–143]. Abrahamse's group published some literatures for LLLT application to stem cells. It is the cellular effect of increasing proliferation and viability that may significantly contribute to the addition of LLLT to the many biomedical disciplines that further augment the successes of regenerative medicine [144]. They reported that low power laser irradiation has been shown to induce adipose-derived stem cell activity by increasing migration, proliferation, and viability, activating protein expression and inducing differentiation in progenitor cells [145–147]. Wu et al. reported that LLLT suppresses inflammatory response of human adipose-derived stem cells by modulating intracellular cyclic AMP level and NF-κB activity [129]. Lipopolysaccharide- (LPS-) induced proinflammatory cytokine expression was inhibited by LLLT and the intracellular cAMP level, which acts to downregulate NF-κB transcriptional activity which was increased. Those results indicate that LLLT can potentially be applied in anti-inflammatory therapy followed by stem cell therapy. We reported that the laser irradiation can direct the extracellular calcification of primary MSCs by altering the intracellular localization of the circadian rhythm protein, CRY1 [2, 3]. Figure 1 presents the beam profile of the laser (wavelength: 405 nm) used in the study (Panel (a)) and the changes in mouse bone marrow mesenchymal stromal cells irradiated for 3 minutes and then cultured for 14 days in osteoblast differentiation medium (Panel (b)) [3]. Alizarin red staining revealed that the stained cells were distributed in a circular area with a diameter similar to that of the laser beam. In addition, the results of immunostaining for CRY1 protein are represented in Figure 2. Whereas CRY1 was distributed across the cytoplasm in control cells, it was localized to the nucleus in cells exposed to laser (wavelength: 405 nm) irradiation. The timing of nuclear accumulation of clock proteins constitutes an important step in the transcription-translation feedback loop driving the circadian core oscillator and is controlled by regulating protein localization and turnover. Our results show that these laser beams promote the nuclear localization of CRY1 and mediate the expression of CRY1 and other proteins downstream of the E-box, which played a critical role in deciding the expression of BMPs [3]. We also reported that laser irradiation suppressed the adipocyte differentiation of mesenchymal stromal cells [2] and accelerated their differentiation into chondrocytes [4]. Abramovitch-Gottlib et al. reported that the consequent phenotype modulation and development of MSCs towards ossified tissue were studied in the combined 3D biomatrix/LLLT system [148]. Their results obtained from the irradiated samples showed enhanced tissue formation, appearance of phosphorous peaks, and calcium and phosphate incorporation to newly formed tissue. Moreover, in irradiated samples ALP activity was significantly enhanced in early stages and notably reduced in late stages of culturing. Those findings of cell and tissue parameters up to 28 days of culture revealed higher ossification levels in irradiated samples compared with the control group. They suggested that both the surface properties of the 3D crystalline biomatrices and the LLLT have biostimulatory effect on the conversion of MSCs into bone-forming cells and on the induction of *ex vivo* ossification [148]. In addition, lasers in visible wavelength were used mostly for LLLT, but the novel laser sources, such as terahertz (THz) laser, were recently investigated for MSCs therapy [135–137]. Alexandrov et al. reported that extended exposure to broad-spectrum THz

Table 1: The effect of LLLT on the MSCs proliferation and differentiation (literatures published in recent 3 years).

Authors	Brief description	Reference
Park et al.	LLLT enhanced angiogenic effect of adipose-derived stromal cells (ASCs) spheroid in hind limb ischemia mice. LLLT is an effective biostimulator of spheroid ASCs in tissue regeneration that enhanced the survival of ASCs and stimulated the secretion of growth factors in the ischemic hind limb.	[126]
Farfara et al.	MSCs were stimulated by LLLT in order to affect neurological behavior and beta-amyloid burden in progressive stages of Alzheimer's disease mouse model.	[127]
Yang et al.	LLLT was applied as an adjunct therapy for MSCs transplantation on the functional recovery of crushed sciatic nerve in rats.	[128]
Wu et al.	LLLT increased the intracellular level of cAMP, which acts to downregulate NF-κB transcriptional activity.	[129]
Nagata et al.	The combination of bone marrow aspirate/LLLT yielded significantly greater bone formation in surgically created critical-size defects in rat calvaria.	[130]
Manuguerra-Gagné et al.	A laser-induced model of open angle glaucoma (OAG) was used to evaluate the potential of bone marrow cell populations and the mechanisms involved in tissue repair. Laser-induced tissue remodeling as a method of targeting effector cells into damaged tissues was also evaluated.	[131]
Lipovsky et al.	The ability of broadband visible light illumination to promote proliferation of MSCs was evaluated.	[132]
Giannelli et al.	The effects of LLLT on mouse MSCs proliferation were investigated underlying cellular and molecular mechanisms, focusing the attention on the effects of laser irradiation on Notch-1 signal activation and membrane ion channel modulation.	[133]
Choi et al.	Adipose-derived mesenchymal stem cells- (ASCs-) seeded acellular dermal matrix was used with LLLT to repair bone defect.	[134]
Alexandrov et al.	Terahertz (THz) laser irradiation of MSCs can cause specific catalytic changes in cellular function that are closely related to the gene expression and differentiation state.	[135–137]
Wu et al.	The change in mRNA expression in rat MSCs after LLLT and the associated molecular mechanisms were investigated.	[138]
Wu et al.	LLLT induced IGF1 expression to promote both the proliferation and osteogenic differentiation of MSCs, whereas it may induce BMP2 expression primarily to enhance osteogenic differentiation.	[139]
Wang et al.	MicroRNA-193 proproliferation effects for bone MSCs were revealed after LLLT through inhibitor of growth family, member 5.	[140]
Soleimani et al.	The influence of LLLT at different energy densities on MSCs differentiation into neuron and osteoblast was examined.	[141]
Saygun et al.	LLLT increased the proliferation of osteoblast cells and stimulated the release of bFGF, IGF-1, and IGFBP3 from these cells.	[142]

radiation results in specific changes in the functionality of cellular DNA. Certain genes in irradiated MSCs cultures are activated, while other genes are repressed. Many of the MSCs genes do not respond to the selected radiation conditions at all, showing that the effect is specific. Additionally, 9 hours of exposure causes significant changes in the MSCs gene expression, while the response to shorter duration (2 and 4 hours) is appreciably less pronounced. Hence, they discussed that the effect of THz radiation was gene and exposure specific and most likely is at the level of DNA transcription [137]. Although each researcher used a different type of laser (i.e., wavelength, power, and pulse-width), MSCs proliferative and differentiation potential can be increased. The mechanisms involved remain to be clarified, but LLLT is a valid approach for the preconditioning of MSCs *in vitro* prior cell transplantation.

4. Conclusion

Regenerative medicine and stem cell therapy have the potential to provide diseases-free, functional tissues and organs, improving the quality of life for patients. They have also the ability to transform the treatment of human disease by introducing combined innovative new therapies such as stem cell therapies and LLLT. Today, researchers are conducting intensive basic and clinical research in the area of laser medicine and photobiology, with the goal of developing new diagnostic and therapeutic modalities. Here, we described some of the latest advances in research on the cellular effects of irradiation with lasers to MSCs. The biological mechanisms underlying such responses significantly differ by the type of laser, target of cells, and other experimental conditions. With the appropriate use of LLLT,

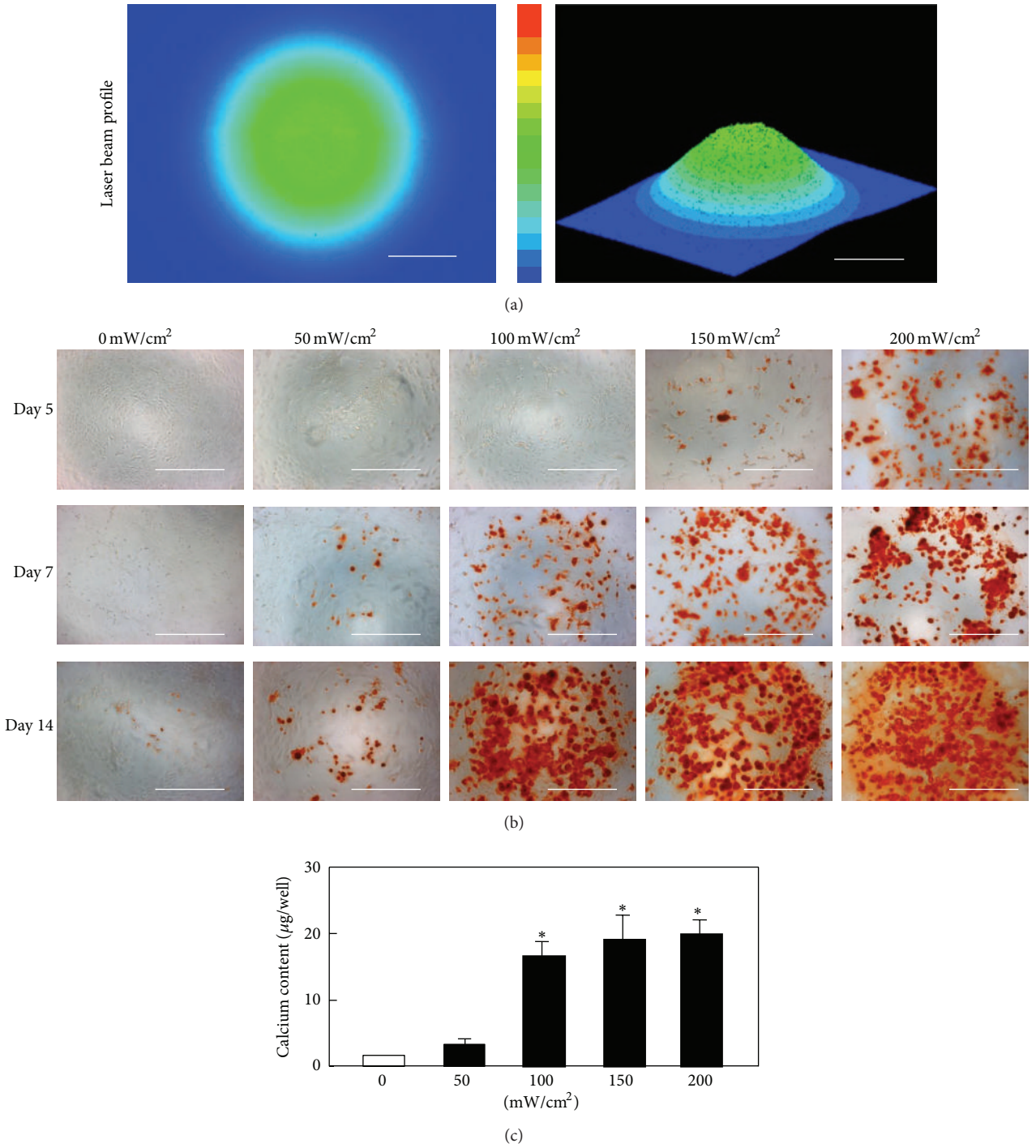

FIGURE 1: (a) The beam profile of the blue laser (wavelength: 405 nm) used in this study (scale bars: 200 μm). MSCs were irradiated for 180 sec at various laser power levels. (b) Alizarin red S staining of irradiated MSCs (magnification 50x; scale bars: 400 μm). After laser irradiation, calcium deposition had increased around the cells in a dose-dependent manner. (c) The quantitative calcium content increased after blue laser irradiation (day 14) relative to nonirradiated cells. Calcium content increases varied with laser energy level ($^*P < 0.01$, indicating significant difference between the calcium content of laser-irradiated MSCs and controls) [3].

the proliferation rate of cultured cells, including MSCs, can be increased, which would be very useful in tissue engineering and regenerative medicine. We must accumulate a systematic knowledge base by carefully analyzing the experimental data currently available, as well as data collected in the future. We believe that light-based biomedical research will open new horizons for photodiagnosis, phototherapy, and MSCs therapies.

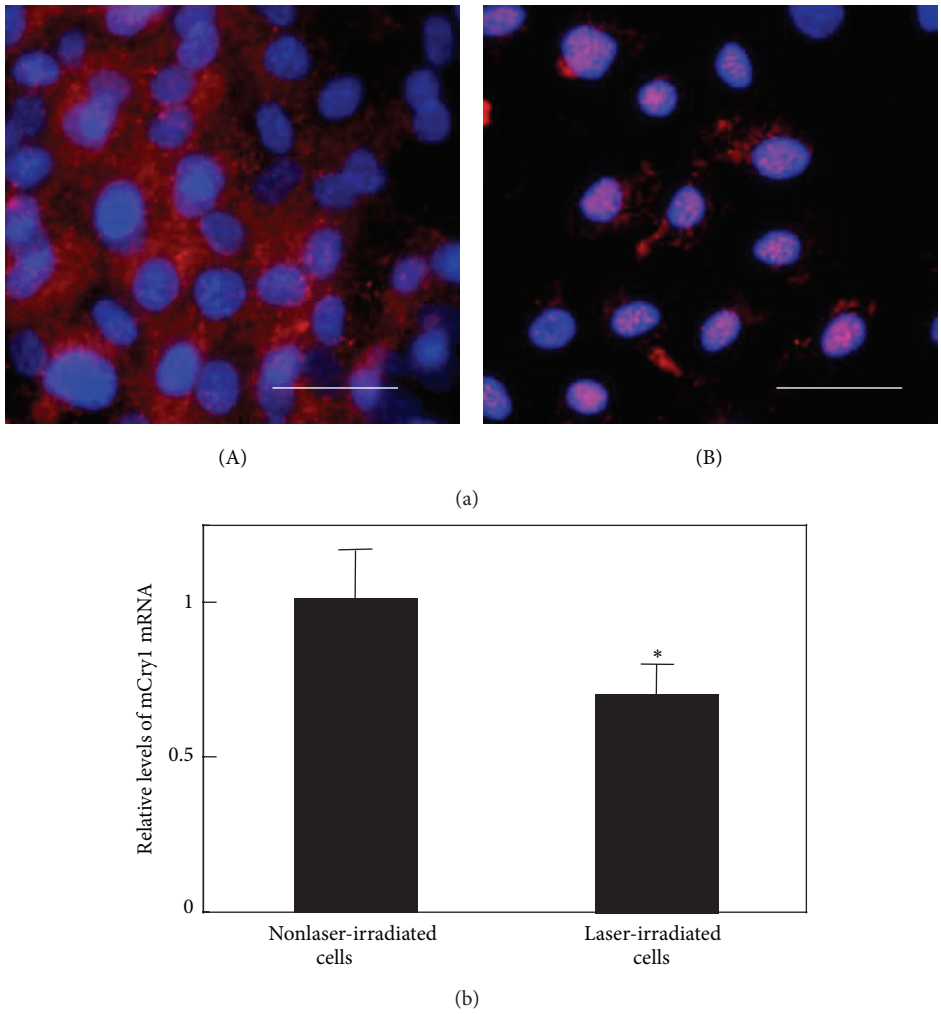

FIGURE 2: (a) Subcellular location of CRY1 proteins in MSCs after laser irradiation (200 mW/cm^2). Cells were double-labeled with DAPI (blue) and CRY1 (red). CRY1 localized to the cytoplasm prior to laser irradiation (A). However, after laser irradiation, CRY1 translocated to the nucleus (B) (scale bars: 50 μm). (b) mRNA levels of Cry1 in MSCs 24 h after laser irradiation (200 mW/cm^2) and in nonirradiated cells. Samples were normalized to mRsp18. The mRNA levels of Cry1 decreased after blue laser irradiation relative to nonirradiated cells ($^*P < 0.01$, indicating significant difference between the relative mRNA levels of laser-irradiated MSCs and controls) [3].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Adult Mesenchymal Stem Cells: When, Where, and How

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Adult mesenchymal stem cells (MSCs) have profound medicinal effects at body sites of tissue injury, disease, or inflammation as either endogenously or exogenously supplied. The medicinal effects are either immunomodulatory or trophic or both. When to deliver these mediators of regeneration, where, and by what delivery apparatus or mechanism will directly determine their medical efficacy. The MSCs help manage the innate regenerative capacity of almost every body tissue and the MSCs have only recently been fully appreciated. Perhaps the most skilled physician-manager of the body's innate regenerative capacity is in orthopedics where the vigorous regeneration and repair capacity of bone through local MSCs-titers is expertly managed by the orthopaedic physician. The challenge is to extend MSCs expertise to address other tissue dysfunctions and diseases. The medicine of tomorrow will encompass optimizing the tissues' intrinsic regenerative potential through management of local MSCs.

1. Introduction

Since the late 1980s when the technology for isolating and culture expanding MSCs was perfected and then reduced to practice in the early 1990s [1, 2], their use for clinically relevant therapies has evolved. Indeed, two very different logics have been proposed and explored. The original logic was that marrow-derived, culture-expanded MSCs, because of their multipotency, could be used in tissue engineering formats to replace injured, damaged, or diseased mesenchymal tissues [3, 4]. Although this logic was pursued for almost three decades and continues to be explored, no product or treatment is currently available. In defense of this pursuit, newer logics and scaffolds now being experimentally tested hold realistic promise for eventual success and clinical use to replace cadaveric products now used routinely.

The documentation that MSCs (perhaps all MSCs) are derived from perivascular cells, pericytes [5, 6], now explains how MSCs can be isolated from almost every tissue in the body [7, 8]. Moreover, the fact that MSCs possess the capacity to secrete immunomodulatory and trophic mediators strongly argues that their natural and normal *in vivo* function is as Medicinal Signaling Cells (MSCs) for sites of injury or inflammation [9, 10] in all of the tissues in which they are housed. Today on the website *clinicaltrials.gov* a search using "mesenchymal stem cells" in the website's search engine shows that over 500+ clinical trials are listed covering a surprisingly enormous array of clinical conditions. All of these clinical conditions have one or both of the immunomodulatory or regenerative (trophic) aspects as central components to the therapeutic intent of using MSCs.

The focus of this treatise is to take the state of knowledge, at *this point in time*, to address the medicinal use of MSCs and to attempt to identify the key parameters to consider for their optimal use in cell-based therapies. In this context, some misconceptions will be addressed since the state of detailed knowledge is relatively small compared to the exuberant expectations of the physicians and scientists consumed by the therapeutic potential of MSCs, the present author included. Thus, this paper is a report on the state of the art of MSCs and it is expected that these new, powerful potential therapeutics will evolve as we have previously witnessed when considering the changes in use and science of hematopoietic and neural stem cells in the last 50 years of their clinical and experimental exploration [11, 12].

2. MSCs

The realization that MSCs are derived from pericytes changes the context of considering how they arise and function *in*

vivo during the body's response to both localized injury and the demand for regeneration/repair. In its simplest inception, the pericyte is released from its association with the basal lamina of the blood vessel situated in the field of injury or inflammation. This released pericyte is exquisitely capable of sensing its surrounding *milieu* and responding by becoming an MSC; this new MSC phenotype becomes activated and keyed to the detailed chemistry and dynamic changes to its local microenvironment. The activated MSCs put out a concentrated localized curtain of bioactive molecules that serves to inhibit the interrogating cells of the body's overaggressive immune system [13, 14]. This is, thus, a first-line defense against the establishment of autoimmune reactions against the injured tissue in the immediate vicinity. In coordination with this protective curtain, the MSC secretes molecules to assist in the establishment of a regenerative (not repair) microenvironment. Included in these trophic mediators are molecules that (A) inhibit ischemia-caused apoptosis; (B) inhibit scar formations; (C) stimulate angiogenesis and vessel stability; and (D) stimulate mitosis of tissue-intrinsic progenitors [15, 16].

The overall effect of locally situated, activated MSCs is to help manage the innate capacity of every tissue to regenerate itself by inhibiting the quick-fix apparatus of scar formation. It is now apparent that the immune system contributes components that not only protect injury sites from "foreign intruders" but also enhance the quick-fix aspects of fill-in with connective tissue that leads to scar. Clearly, in embryos where the immune system has not developed, scarless healing is quite normal [17]. Likewise, in neonates, the scarless regenerative capacity is substantial. As animals get larger and as they age, the vascular density in various tissues decreases and tissue regeneration, or even repair, becomes logistically different [18]. The key to the MSCs' clinical efficacy is the fact that every living tissue turns over. This means that as cellular and extracellular matrix (ECM) components expire, they are replaced by similar components. The innate regenerative capacity of a tissue is tied to this turnover dynamic. For example, the fact that bone is resorbed and fabricated in a coupled cellular mechanism allows fractures to heal at a rate directly linked to the natural, age-related ratio of fabrication to resorption, that is, rapid healing in young growing subjects and very slow healing in older, osteoporotic subjects [19, 20]. This begs the question of whether it is a youthful microenvironment (i.e., molecular) that controls turnover/repair or whether it is the cells themselves that provide the dynamic queuing.

If MSCs are, indeed, the managers of site-specific tissue regeneration, their presence, their numbers, their proper activation, and their coordinated and dynamic function can have a profound impact on injury and disease progression. The medicinal activity of MSCs is, thus, dependent on aspects of the management of the tissue and the site of injury or disease with respect to the therapeutic capacity of either endogenous or exogenously supplied MSCs. This infers that MSCs are intrinsically curative and that their therapeutic effectiveness solely depends on the "when, where, and how" of their delivery or presentation at sites of injury, disease, or regeneration.

3. When

At the site of any tissue injury, large or small, there is an immediate trigger to the acute inflammatory response which serves to bathe the site with molecules and cells to protect against invasion by toxic molecules or foreign organisms. This acute inflammation serves to also condition the site for either regeneration, repair, or scarring. The presence of MSCs following this initial flushing of the injury site would inhibit the intrusion of immune interrogating cells and further protect the site from the disbursement of agents that could be toxic to resident tissue cells. The activated MSCs function to inhibit connective tissue cells from pumping out massive amounts of collagen and other components that function as both the soil and the bed for scar. Thus, early in the injury response, sufficient numbers of MSCs could naturally serve to protect the injury field from degenerate events and allow regenerative repairs to be initiated. In this regard, in an aging individual with decreased numbers of MSCs, scarring would be more prominent.

Given the above logic, the "when" to deliver MSCs is after the major acute inflammation has died down, relatively early after the injury event. This could be at 48 hours after an acute myocardial infarct or by day 7 following a stroke as observed in preclinical animal models [14–19]. If the injury or disease state is chronic, multiple presentations of MSCs, say twice per week for 4 weeks (the Osiris Therapeutics, Inc., protocol for Crohn's Disease), anticipate multiple events and an extended duration of MSC exposure. In extreme cases such a heavily scarred tissue such as observed in patients with COPD or chronic asthma [21], again multiple exposures suitably spaced from one another should be required.

The issue of scarred tissue is quite complex and the age and health status of the subject are critical. Scar is a living tissue composed of massive ECM and its maintenance cells. The assumption is that scar, say in the lung, turns over. If MSCs do indeed function, either inhibiting the formation of scar or inhibiting the entrance or development of scar forming cells, then MSCs must reside at sites of scar for a considerable length of time or appear at critical intervals to inhibit scar formation or expansion while providing a microenvironment for the afflicted tissue to regenerate itself. In an animal model of asthma, multiple exposures to MSCs are required to enhance scar turnover and its eventual elimination [21].

4. Circulating or Mobilizing MSCs

The best data available indicates that MSCs do not circulate [22, 23]. Indeed, when MSCs were infused into the venous system of one arm only, a few MSCs could be detected right after infusion in the blood of the other arm, but none thereafter [22]. It is important to understand that if 100 million MSCs are slowly infused into the blood stream of an adult (even if all of these MSCs circulated which is improbable), the number of circulating blood cells is in such vast excess that it would almost be impossible to detect even one MSC by cell-sorting or by colony formation (MSC adhesion to culture dishes in optimal plating medium) [24].

This also begs the question as to when and if MSCs can be mobilized to sites of injury. The entire concept of "mobilization" stems from a misconception and faulty word-usage in hematology. It is commonly accepted to call the action of the drugs G- or GM-CSF as "mobilizing" because huge amounts of hematopoietic progenitors can be detected in peripheral blood samples [25, 26]. These drugs cause massive cell proliferation in bone marrow and the progeny becomes so densely packed that they push out through the sinusoids into the blood stream. This is a cell crowding event not cell-specific mobilization. Likewise, if rodents are grown in chambers of low oxygen, MSCs can be found in circulating blood consistent with an increase in blood levels of HIF-1α [27]. I believe the circulating MSCs are present because of numerous blood vessel breaks and the release of pericytes from their basal lamina anchorage not because of HIF-1α causes the mobilization of the cells.

The recent report that no circulating MSCs could be detected in various patients with chronic diseases but could be detected in patients with multiple fresh fractures does not disprove the concept that MSCs can circulate and can be mobilized [24]. The blood sample of the chronic disease patients contained no MSCs because the initiation of the chronic condition had long since passed and the micro "injury" to sustain a chronic condition is not known and difficult to time. Moreover, the sensitivity of a cell-sort or colony plating scheme is too low to detect MSCs if, indeed, they were mobilized and circulating. Again, for emphasis, the pericyte is released from its tether in the basal lamina at injury or inflammation to become an MSC that is both mobile and it can be swept into the blood stream. More basic information is required to understand these events *in situ* before we discard the notion that MSCs can be mobilized or that they circulate. The data involving SDF-1 (discussed below) could be used to argue that MSCs are motile and dock in specific regions of the vascular tree.

5. Where

MSCs function at sites of blood vessel damage or inflammation. That is where they need to be delivered. This can be accomplished by systemic delivery, but it is clear that these exogenous MSCs are fragile and can be eliminated almost immediately upon entering the blood stream [28]. Likewise, they can irreversibly lodge in the lung and liver [29] and, thus, never reach the tissue target. Therefore, where exogenous MSCs are introduced in the body can have a profound influence on their capacity to reach sites of recent or current injury or inflammation. This issue of "where" to infuse MSCs has been exquisitely documented by Lin et al. who introduced MSCs into mice via a carotid cutdown using a stiff catheter into the aortic arch and, thus, into the left ventricle and descending aorta whose blood flow bypasses the lung and liver for at least one full body passage [30]. These experiments were done in a mouse in which one leg was irradiated causing a marrow injury 4 hours prior to luciferase-labeled MSC infusion. The standard tail vein infusion uses one million MSCs while left ventricle infusion could deliver 10 times less yet document that the labeled MSCs did indeed dock in the injured leg marrow.

Direct injections of MSCs into synovial joints, spinal disc, or intramuscular are also being used clinically with apparent success. The most detailed study has involved a cork-screw catheter into an infarcted heart [31]. Penn and colleagues have shown that the infarcted rodent hearts released SDF-1 and that if exogenous MSCs are delivered [32, 33] within 48 hours after injury the MSCs will dock in this tissue and protect the heart from the subsequent damaging events. Importantly, if MSCs are introduced systemically on day 7, the SDF-1 is no longer being secreted and MSCs will not dock. Moreover, by using a plasmid for SDF-1 and delivering it to damaged heart, the SDF-1 subsequently produced will serve as a powerful chemoattractant for MSCs, presumably from marrow and other depots, to attract them to the injured tissue and to assist in both the protection and the recovery of the heart tissue [34, 35]. The sustained secretion of SDF-1 also holds promise for treating patients with chronic heart issues and is part of a current clinical trial (http://www.juventasinc.com/index.html).

Last, although systemic and direct injections of MSCs into afflicted tissue are in use, the introduction of MSCs into the peritoneal cavity has never been properly evaluated, especially for Crohn's disease, inflammatory bowel disease, or ailments of the abdominal region. Since the lymph tree in this cavity is so prominent, it is tempting to propose that MSCs might be highly effective if introduced into this tree. Likewise, would this tree be a useful port for systemic introduction of exogenous MSCs?

It must be emphasized that there is no quantitative information that elaborates the number of "docked" MSCs as related to a specific therapeutic outcome. The initial intravenous doses of MSCs are extraordinarily large in both rodent-disease models and in clinical trials where 1–5 million MSCs/kg are the standard doses. Moreover, although docking strategies have been employed, the efficiency of docking and the potency of MSCs are difficult to quantitate and almost impossible to relate to the composite therapeutic outcomes. As inferred above, if MSCs must dock in the damaged heart tissue and in the servicing lymph tree, the question of efficiency of docking and potency of MSCs becomes even more difficult.

6. How

Although clinical trials are now in play in which MSCs are mostly delivered intravenously, intramuscularly, and into the synovial joints, there are other routes of administration that are being explored. The cork-screw catheter was used to increase the needle path (creating an increase in focal injury) and to maximize the retention of MSCs in the heart and thus delivers MSCs into afflicted cardiac tissue where the MSCs not only dock in and on this newly injured tissue, but also spill out into the circulation [36, 37]. It may be that this spillage allows the MSCs to dock in the lymph system that services the heart where they may affect the local immune system (my speculation).

A very unusual, but potentially important delivery route has been published indicating that the upper sinus might be a perfect routing to the brain. Currently, intrathecal administration of MSCs is being used for patients with MS or ALS. Cells or drugs like insulin delivered to the upper sinuses are captured by a liquid stream that flows from around sensory axons of olfactory nerves up into the extracellular fluid that courses through in the brain from front to back [38, 39]. This may also be a more logical pathway for patients suffering from Parkinson's disease or MS to receive therapeutic cells including MSCs as has been published in rodents [40].

The therapeutic effect achieved by MSCs is by producing a spectrum of bioactive molecules that affect the injury site by both trophic and immunomodulatory mechanisms. The question arises as to whether, by priorly exposing MSCs to specific agents in culture, the paracrine activities could be optimized for a specific therapeutic outcome. For example, pretreatment of MSCs with IFN-γ protects [41] against graft-versus-host-disease (GVHD). Importantly, MSCs (unpretreated) mount an immunomodulatory assault on GVHD and two MSC products have been approved for use in children with steroid-refractory GVHD with substantially positive outcomes. Will IFN-γ pretreated MSCs eliminate all GVHD? This is doubtful given the complexities involved. However, if such pretreatment eliminated a sizeable proportion of GVHD upon bone marrow transplantation, this could save many lives and decrease the huge hospital costs.

Last, since cultured, exogenous MSCs are delicate and susceptible to damage upon entering the blood stream [28] or by direct injection into tissues; the encapsulation of MSCs may be a preferred route of administration with their subsequent slow release. For example, a small private company in Italy called Lipogems (for whom I currently consult) has an apparatus for treating lipoaspirate and generating 500 micron aggregates of adipocytes with MSCs trapped inside [42]. These aggregates when introduced into culture do not plate out, but MSCs can be observed to crawl out onto the plate after 4–7 days. Such autologous MSCs would appear at sites of injury after the acute inflammatory phase of their introduction and could be then highly effective. Clinical use for fecal incontinence, osteoarthritis, muscle injury, and so forth has been reported to be highly effective. Proper double-blind, placebo control clinical trials should be quite interesting for this MSC slow release and protective technology.

7. Who Makes the Therapeutic Molecules?

Because MSCs function medicinally at sites of injury, it is assumed that they produce a spectrum of therapeutically active molecules. But, do they? An ingenious experiment has been performed by Adonis Hijaz, MD, and his colleagues [43] (Hijaz et al., personal communication). A urinary incontinence model is generated in rodents by placing a balloon in the animals' vagina. The urethra is injured causing leakage of urine that can be quantitatively accessed by leak-point pressure. If human MSCs labeled with a fluorescent dye are introduced into the urethra, the animal is back to normal by day 4. If, on day 1, the animal is sacrificed and the urethra sectioned, laser capture microscopy can isolate tissue containing the fluorescently tagged MSCs and tissue situated next to the labeled hMSCs. In a separate injured animal, the injured tissue that has never been exposed to hMSCs can be isolated by laser capture techniques. RNA chips using purified RNA from these laser captured specimens indicate that the hMSCs are making many different molecules compared to what they originally made on the Petri dish from which they were expanded and isolated. More interesting is the fact that injured tissue situated next to the hMSC is making over 90 different molecules compared to the injured tissue that had never been exposed to the hMSC. By using both rodent specific RNA chips and human specific RNA chips, the question can be asked at that one time point: who is making the therapeutically relevant molecule the rodent host tissue or the hMSC? A more detailed temporal analysis will be needed to not only answer this question, but establish the dynamic interaction between the hMSC and the injured tissue. Having stated this question related to the source of secretion of the therapeutic molecules, the introduction of MSCs acts to inhibit scarring and stimulate *de novo* regeneration [15, 16].

The reason for reviewing the above is to emphasize the emerging theme that MSCs appear to be assisting the host tissue to maximize its *intrinsic* regenerative capacity. The local management of the immune cells and the tissue specific progenitors appears to be accomplished by very few, locally situated, and short-lived MSCs. This innate regenerative capacity of almost every host tissue has never been properly managed except, perhaps, in orthopedics where the vigorous regenerative and repair capacity of bone (maybe through local MSCs) is managed by orthopedic physician interface.

The Medicine of Tomorrow may be the management of MSCs to optimize the body's very powerful and ever-changing intrinsic regenerative potential.

Conflict of Interests

The author declares that Case Western Reserve University receives royalties from Osiris Therapeutics some of which are shared with him to cover his formation of Osiris Therapeutics and patents transferred out of the university.

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Review Article

Regenerative Translation of Human Blood-Vessel-Derived MSC Precursors

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Mesenchymal stem/stromal cells (MSCs) represent a promising adult progenitor cell source for tissue repair and regeneration. Their mysterious identity *in situ* has gradually been unveiled by the accumulating evidence indicating an association between adult multipotent stem/progenitor cells and vascular/perivascular niches. Using immunohistochemistry and fluorescence-activated cell sorting, we and other groups have prospectively identified and purified subpopulations of multipotent precursor cells associated with the blood vessels within multiple human organs. The three precursor subsets, myogenic endothelial cells (MECs), pericytes (PCs), and adventitial cells (ACs), are located, respectively, in the three structural tiers of typical blood vessels: intima, media, and adventitia. MECs, PCs, and ACs have been extensively characterized in prior studies and are currently under investigation for their therapeutic potentials in preclinical animal models. In this review, we will briefly discuss the identification, isolation, and characterization of these human blood-vessel-derived stem cells (hBVSCs) and summarize the current status of regenerative applications of hBVSC subsets.

1. Introduction

Adult multipotent stem/progenitor cells are promising cell sources for tissue repair and regeneration because of their self-renewal, differentiation capacity, and secretion of trophic factors [1]. Though developmentally not as versatile as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), adult stem/progenitor cells represent a more clinically relevant cell source for regenerative medicine due to less ethical and/or safety issues [2]. In particular, mesenchymal stem/stromal cells (MSCs) and MSC-like multilineage precursor cells, including adipose-derived stem cells (ADSCs), mesoangioblasts, and multipotent adult progenitor cells (MAPCs), have attracted significant clinical attentions, largely owing to their accessibility as well as the robust trophic and immunosuppressive functions.

It has been more than a decade since the first discovery of MSCs and similar precursor cells in human bone marrow (BM), adipose, placenta, and many other tissues [3–5]. Typical MSCs are plastic-adherent and expressing cell surface

markers such as CD29 (integrin-β1), CD44 (hyaluronic acid receptor), CD73 (ecto-5′-nucleotidase), CD90 (Thy-1), CD105 (endoglin) but negative for CD14 (myeloid cell-specific leucine-rich glycoprotein), CD31 (PECAM-1), CD34 (hematopoietic/stem/endothelial cell marker), and CD45 (pan-leukocyte marker), in addition to the capacity of differentiating into common mesodermal cell lineages including osteoblasts, chondroblasts, and adipocytes. However, BM titers of human MSCs decline significantly with age [6]. Recently, clonally derived MSCs injected intra-arterially have been shown to selectively engraft at perivascular locations in the BM sinusoids/microvessels following a localized radiation injury in the mouse hindlimb [7]. Engrafted MSCs can not only proliferate locally in the long-term but also be serially transplanted into the secondary host while maintaining similar homing and engraftment efficiency. These results imply that BM can be therapeutically exploited as a renewable source for exogenous MSC transplantation.

Despite the extensive investigation, the native identity of MSCs has long been obscured by the retrospective identification method in culture. Recently, there is increasing evidence indicating the relationship between tissue-specific stem/progenitor cells and vascular/perivascular niches [8, 9] as well as the presence of multipotent stem cells of vascular origin [10–12]. Sacchetti et al. demonstrated that CD146+ subendothelial stromal cells residing on the BM sinusoidal wall not only self-renew and display vascular mural cell features but also form bone and establish the hematopoietic microenvironment [13]. Consequently, a hypothesis that blood vessels throughout the human body serve as a reservoir of multipotent precursor cells has been formulated [14, 15].

Using immunohistochemical assays and fluorescence-activated cell sorting (FACS), we and other researchers have prospectively identified and purified three populations of multipotent precursor cells from human blood vessels: myogenic endothelial cells (MECs), pericytes (PCs), and adventitial cells (ACs). Collectively named human blood-vessel-derived stem cells (hBVSCs), these subpopulations of hBVSCs can be isolated from blood vessels within human skeletal muscle, with each expressing a unique profile of cell surface antigens. PCs and ACs have also been isolated from several other human tissues such as fat and lung [16–19]. MECs, PCs, and ACs all possess common mesodermal multipotency and display typical MSC markers, suggesting their contributions to the heterogeneous MSC entity.

2. Native Distribution of Human Blood-Vessel-Derived Stem Cells

Human blood vessels typically consist of three definitive structural tiers: tunica intima, tunica media, and tunica adventitia [20]. Tunica intima is the innermost layer of a blood vessel and primarily composed of endothelial cells (ECs), supported by an abluminal layer of elastic fibers. Tunica media contains multiple layers of smooth muscle cells (SMCs) while tunica adventitia, the outermost layer, consists of extracellular matrix (ECM), fibroblast-like stromal cells, and vasa vasorum. At the microvessel (arterioles and venules) and capillary level, the construction of vessels is reduced to only endothelial cells and surrounding vascular stromal cells (VSCs), that is, pericytes. The EC-to-PC ratio ranges from 100 : 1 to 1 : 1 while PC coverage of abluminal EC surface varies from 10% to 50%, depending on the tissue origin [21]. Increasing evidence indicated the existence of pericyte-like cells in normal intima, suggesting a heterogeneous PC network within blood vessels of all sizes [22, 23]. Moreover, comparative characterization studies have demonstrated differences in expression of cell lineage markers, developmental potentials, and angiogenic capacity among human VSCs derived from the walls of blood vessels of different sizes (artery, vein, and microvessel) [24]. These results imply innate differences between VSCs residing in different vascular structural tiers and tissue-dependent divergence of VSCs.

Developmentally myogenic cells (MCs) and ECs of the vertebrate limb may derive from a common somitic precursor [25]. Notch signaling has recently been shown to play an important role in the selection of endothelial versus myogenic cell fate in multipotent somitic Pax3+ cells before their migration to the limbs during embryonic development [26]. Previous studies showed that cells coexpressing MC and EC markers reside within the interstitial space of skeletal muscle and possibly contribute to postnatal muscular development [27]. Consequently we hypothesized that an intermediate cell type coexpressing MC and EC markers exists within the interstitium of postnatal skeletal muscle, presumably associated with blood vessels and potentially multipotent. Indeed, immunohistochemistry revealed that a rare subset of myogenic precursor cells coexpresses MC and EC markers at the microvascular level [28]. These myogenic endothelial cells (MECs) not only express MC markers including Pax7 and CD56 but also display EC markers including CD34, CD144 (VE-cadherin), von Willebrand factor (vWF), and *Ulex europaeus* agglutinin-1 (UEA-1) [28].

Pericytes (PCs) are commonly regarded as a structural component of small blood vessels that regulate vascular contractility, stability, and integrity [29–31]. PCs also modulate EC proliferation/vascular remodeling and are involved in specialized vascular functions including blood-brain barrier and renal tubulovesicular coordination as well as several pathological conditions [21, 23, 32–35]. However, this particular cell population has not been well defined in most of the human organs due to a lack of representative cell marker(s). We previously described microvascular PCs in multiple human tissues based on robust expression of CD146 (Mel-CAM), NG2 (chondroitin sulphate), platelet-derived growth factor receptor-beta (PDGFRβ), and the absence of myogenic (CD56), hematopoietic (CD45), and endothelial cell surface markers (CD31, CD34, CD144, and vWF) [16]. Alkaline phosphatase (ALP) is another marker used to typify PCs in human skeletal muscle [36, 37]. Alpha-smooth muscle actin (α-SMA), on the other hand, can be detected in PCs encircling arterioles and venules but not in those surrounding most capillaries [16]. PCs *in situ* also express classic MSC markers: CD44, CD73, CD90, and CD105 [16].

Adventitial cells (ACs) have been perceived as fibroblast-like cells producing adventitial ECM, a loose structural

element enclosing media of arteries and veins. Recent studies indicated that CD34 identifies 2 concentric rings of cells residing in intima and adventitia, respectively [38]. Specifically, the CD34+/CD31−/CD45−/CD146− cell subset localized within adventitia, distinct from typical CD34+ endothelial progenitor cells (EPCs), was shown to possess stem/progenitor cell properties and actively participate in vascular pathophysiology [39, 40]. In a vascular injury model, ACs initiated a remodeling process by proliferating and migrating into media and intima and further differentiated into smooth muscle cells, suggesting the importance of adventitia in vascular cell trafficking and blood-vessel remodeling [41, 42]. Furthermore, ACs located in the "vasculogenic zone," that is, the interface between tunica media and adventitia, have been described as precursors endowed with the capacity to differentiate into endothelial cells and participate in the blood-vessel formation as well as the pathogenesis of atherosclerosis [42–44]. Similar to PCs, there is increasing data suggesting a wide distribution of CD34+ perivascular stromal cells, even at the microvascular level [45].

3. Purification of Human Blood-Vessel-Derived Stem Cells

Based on the cell surface marker expression identified by immunohistochemistry, we discovered a unique combination of surface antigens for each subset of hBVSCs that allows one to purify these cells to homogeneity through FACS: MECs (CD34+/56+/144+/45−), PCs (CD146+/34−/45−/56−), and ACs (CD34+/31−/45−/56−/146−) [16, 18, 28]. The isolation and purification of hBVSC subpopulations have been well established [46]. The workflow of hBVSC purification from fresh human skeletal muscle biopsy is illustrated in Figure 1. To date, skeletal muscle is the only human tissue that has been shown to contain all three hBVSC subsets, with MECs not yet identified in other adult human organs. To isolate PCs and ACs from human adipose, fresh biopsy or lipoaspirate is dissociated mechanically and enzymatically to obtain stromal vascular fraction (SVF), followed by similar cell labeling and sorting processes [16, 18, 47]. PCs can also be purified from human placenta, pancreas, skin, heart, and other organs following a similar protocol [16, 48]. ACs, on the other hand, can be isolated from other human tissues including lung and BM or directly from blood vessels such as saphenous vein [19].

MECs exist at a very low frequency (<0.5%) within the vasculature of human skeletal muscle [28]. On the contrary, PCs can be found in many human tissues at different proportions, for example, 0.29 ± 0.09% in adult skeletal muscle, 0.88 ± 0.18% in fetal skeletal muscle, 0.65 ± 0.10% in adult pancreas, 1.68 ± 0.78% in placenta, and 1.21 ± 0.52% in myocardium [16, 17, 48]. Adipose SVF contains higher frequencies of PCs (14.6 ± 1.02%) due to the abundance of microvasculature in human fat and enrichment of vessel-associated cells during the isolation procedure [16]. On the other hand, ACs represent 9.8 ± 1.7% of the intact adipose SVF and up to 23.8% of SVF from human lipoaspirate for the same reason [18, 47]. In contrast, we found only 2.70 ± 1.01% ACs in human skeletal muscle (unpublished data). Further investigation is needed to determine whether the native frequency of each hBVSC subset changes with multiple physiological parameters, such as age and gender, and/or pathological conditions.

4. Characterization of Human Blood-Vessel-Derived Stem Cells

After FACS purification, hBVSCs subpopulations can be either examined/utilized freshly or further expanded in culture [16–18, 47]. MECs, PCs, and ACs have been independently shown to possess mesenchymal differentiation capacities including chondrogenesis, osteogenesis, adipogenesis, and skeletal myogenesis *in vitro* and express classic MSC markers such as CD44, CD73, CD90, and CD105 natively or in culture [16, 18, 28, 36, 49].

MECs not only proliferated at a significantly higher rate than sorted CD56+ MCs and CD34+/144+ ECs, even in low-serum culture conditions, but also were more resistant to cell death when cultivated under oxidative stress (400 μM H_2O_2) [28]. PCs, freshly sorted or long-term cultured, from multiple tissues including adipose, placenta, and pancreas exhibited a similar level of skeletal myogenesis *in vitro* as muscle-derived PCs, suggesting a generalized myogenic potential of PCs in the human body [16]. In addition, PCs displayed strong chemotactic response toward papain/pepsin digested ECM harvested from porcine urinary bladder and formed capillary-like structures with/without ECs in two- and three-dimensional Matrigel cultures/cocultures [16, 50]. Similar to PCs, ACs derived from different tissue origins showed the same phenotype and robust mesodermal developmental potentials, suggesting that MSCs can be derived from an alternative systemic source which is distinct from PCs [18]. In regular culture, ACs did not express any of the cultured PC markers including NG2, α-SMA, CD146, and PDGFRβ but shared the expression of vimentin with PCs [18]. The cellular kinetics of hBVSC subsets was recently reviewed in [51].

To further investigate whether hBVSC subsets meet the criteria of *bona fide* stem cells, we obtained clones of MECs, PCs, and ACs through either FACSAria autoclone system or limiting dilutions [16, 18, 28, 49]. At the clonal level, all three hBVSC subsets indeed exhibited typical MSC markers as well as robust mesenchymal differentiation capacities in culture [16, 18, 28, 49]. Consequently we theorized that hBVSC subsets are genuine ancestors of MSCs. Interestingly, it has been shown that ACs proliferate significantly faster than PCs and partially express aforementioned PC markers following treatment of angiopoietins-2 or angiotensin-II, suggesting adaptation of PC phenotypes and/or differentiation into PC-like cells upon stimulation [18]. Therefore it is speculated that ACs serve as the progenitor of pericytes [18, 52]. Nevertheless, future studies with appropriate cell lineage tracking will be required to convincingly establish the hierarchy and developmental relationship among hBVSC subpopulations and between various vascular/perivascular cell populations [53].

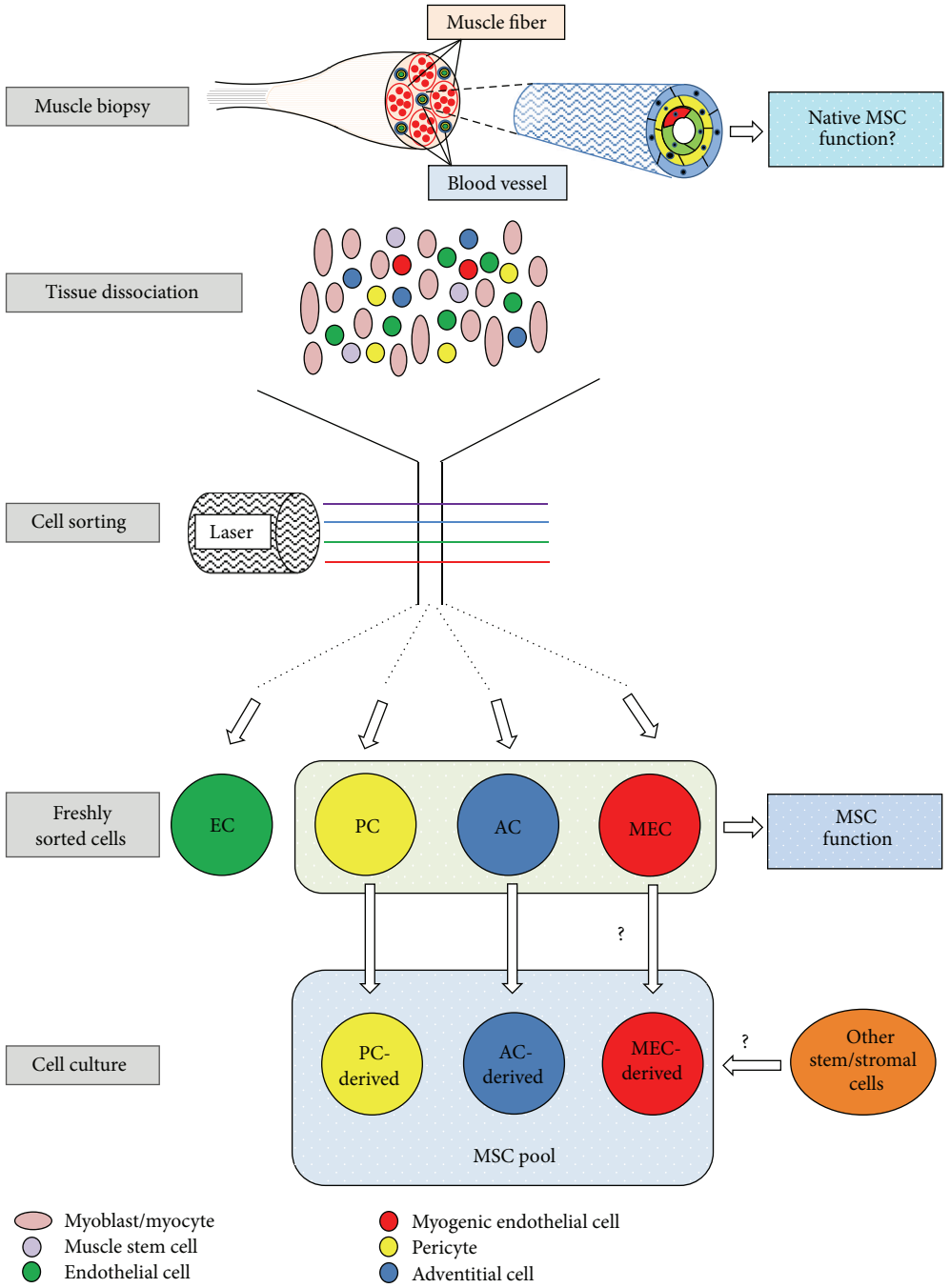

Figure 1: Schematic depiction of hBVSC purification from human skeletal muscle biopsy. Vascular/perivascular cells residing in the blood vessels within the interstitial space of human muscle fibers include endothelial cells (ECs, green), myogenic endothelial cells (MECs, red), pericytes (PCs, yellow), and adventitial cells (ACs, blue). All cells, including immature/mature myocytes and muscle stem cells (i.e., satellite cells), are mechanically and enzymatically dissociated from fresh muscle biopsy. Dissociated cells are subsequently purified to homogeneity by fluorescence-activated cell sorting (FACS). Newly sorted MECs, PCs, and ACs readily exhibit multilineage developmental potentials. Purified PCs, ACs, and possibly MECs give rise to authentic MSCs in long-term culture. However, whether other stem/stromal cells participate in the MSC entity remains to be tested. Moreover, whether native hBVSCs function as typical MSCs *in situ* and/or actively repair/regenerate defective tissues require further investigation.

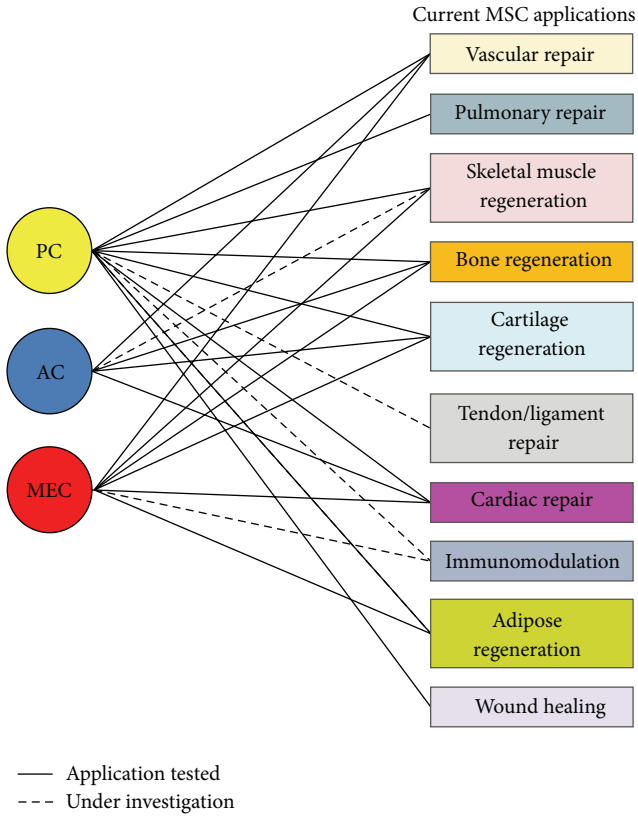

Figure 2: Potential translational applications of hBVSC subpopulations. The current translational applications of typical MSCs are summarized on the right. The current status of translational research for each hBVSC subset is outlined, whether the specific application has been tested or is presently under investigation.

5. Regenerative Applications of Human Blood-Vessel-Derived Stem Cells

As we described above, each hBVSC subset has been shown to display stem cell characteristics and exhibit mesodermal multipotency upon appropriate induction. In addition, all three hBVSC subpopulations have been demonstrated to serve as robust paracrine units, even under stress conditions [50, 52, 54]. However, the relative significance of paracrine function, direct differentiation, and cellular interaction by individual hBVSC subtype may depend on specific pathological conditions and corresponding regenerative actions. Figure 2 summarizes putative applications of all three hBVSC subpopulations in regenerative medicine. A number of representative applications will be discussed in detail below.

5.1. Skeletal Muscle Regeneration

5.1.1. Myogenic Endothelial Cells. Because of their high myogenic potential *in vitro*, MECs have been tested for muscle regeneration in immunodeficient mouse models of cardiotoxin-induced muscle injury and Duchenne muscular dystrophy [28]. The superior myogenic capacity of MECs over typical CD56+ MCs, CD34+/144+ ECs, and unpurified human primary skeletal muscle cells (hPSMCs) was demonstrated by substantially more regenerating human spectrin-positive myofibers after intramuscular injections into cardiotoxin-injured mouse hindlimb muscles [28]. Moreover, to clarify whether newly regenerated human myofibers originated only from the differentiation/fusion of donor cells or in fact involved the participation of host cells, *in situ* hybridization with mouse X chromosome-specific probe was performed. None of the nuclei within the human spectrin-positive myofibers were identified by the mouse-specific probe, confirming their solely human origin [28]. In addition, regeneration of myofibers coexpressing dystrophin and human lamin A/C was observed in dystrophic mouse muscles after injections of human MECs in mdx/SCID mice [28]. Intriguingly, MECs can also be identified/isolated in nonvertebrates such as leech and directly participate in myogenesis *in vitro* and *in vivo*, similar to their vertebrate counterparts [55]. Recently, the murine counterpart of human MECs was shown to play a role in skeletal muscle homeostasis, inhibiting intramuscular adipogenesis through cell-autonomous and cell-cell interactive mechanisms with active Bmpr1a signaling [56]. Together these data suggest that MECs remain an evolutionarily conserved, distinct myogenic precursor population which actively participates in muscle homeostasis and regeneration and possibly bridges MC and EC during muscle development.

5.1.2. Pericytes. The application of PCs in muscle regeneration was examined in immunodeficient mouse models of cardiotoxin-induced muscle injury and Duchenne muscular dystrophy [16, 17, 36]. Freshly sorted or cultured PCs from human muscle and adipose were injected directly into the cardiotoxin-injured gastrocnemius muscles of SCID/NOD mice [16]. PCs from either tissue source regenerated more human spectrin-positive myofibers than purified MCs or ECs, indicating the authentic myogenic capacity of PCs [16]. Similarly, injections of placental PCs into dystrophic gastrocnemius muscles in SCID/mdx mice not only yielded more dystrophin-positive myofibers but also increased the number of local vWF-positive microvasculatures [17]. The human origin of regenerating myofibers was confirmed by *in situ* hybridization with human-specific probe, coexpression of human lamin A/C, or GFP-based cell tracking [16, 17]. Dellavalle et al. further demonstrated that, through ALP-based cell lineage tracking, native PCs residing in the skeletal muscle participate in the postnatal myofiber development, especially under pathological conditions, and contribute to the satellite cell compartment [37]. These results reflect the robust myogenic potential of PCs *in vivo* that can be generalized to PCs from more clinically accessible nonmuscle tissues like adipose and placenta.

Nevertheless, not all PCs within the skeletal muscle are myogenic and contributing to muscle formation. Birbrair et al. reported the presence of two subtypes of PCs within the skeletal muscle: the adipogenic Nestin−/NG2+ (type-1) and myogenic Nestin+/NG2+ (type-2) PCs [57]. Only type-1 PCs expressed PDGFRα, an adipogenic progenitor marker, and contributed exclusively to fat deposition but not

myofiber formation after muscle injury [57]. Future research is needed to determine whether successful muscle regeneration depends on a dynamic balance between myogenic type-2 and nonmyogenic type-1 PCs and/or other myogenic and nonmyogenic stem/progenitor cells [58]. Additionally, whether PDGFRα+ adipogenic PCs contribute to pathological fat accumulation in myopathies and muscle ageing and their relationship with mesenchymal fibro-adipogenic precursors requires further investigation [59]. These results also imply that separation of subtypes of muscle PCs may be necessary to increase the myogenic efficacy of PC treatment in injured/diseased muscle.

5.2. Vascular Regeneration

5.2.1. Myogenic Endothelial Cells. MECs have been shown to form capillary structures in Matrigel culture and implanted Matrigel plugs, suggesting that MECs retain their vascular traits and angiogenic properties *in vitro* and *in vivo*, even after long-term culture [49]. Another vascular progenitor cell (VPC) population coexpressing endothelial and myogenic cell markers (CD34+/133+/KDR+/desmin+) has been derived from human fetal aorta [60]. Upon being transplanted into ischemic muscles, these myogenic VPCs not only alleviated the symptomatic outcome but also incorporated into regenerating myofibers and microvessels in a murine model of hindlimb ischemia [60]. Altogether these studies indicate reparative and regenerative capacities of MECs for angiogenesis and revascularization.

5.2.2. Pericytes. To investigate their capacity for vascular repair, PCs were seeded onto small-diameter, bilayered elastomeric poly(ester-urethane)urea scaffolds and incubated in bioreactors for 2 days before being implanted as aortic interposition grafts in Lewis rats for 8 weeks [61]. PC-seeded grafts showed a significantly higher patency rate (100%) than unseeded controls (38%) and exhibited extensive tissue remodeling including elastin/collagen deposition, multiple layers of α-SMA- and calponin-positive cells, and a monolayer of vWF-positive cells in the lumen, indicating the potential of PCs in vascular repair and tissue engineering [61]. Nonetheless, contrary to their typical angiogenic role in tissue repair, PCs were recently demonstrated to inhibit microvessel formation and further induce microvessel dissociation through CXCR3-induced involution of ECs in an *in vitro* angiogenic model [62]. These results suggest multifaceted regulatory functions of PCs in vascular repair/regeneration, not only promoting angiogenesis/vasculogenesis but also contributing to the pruning of excessive/immature microvessels during tissue repair.

5.2.3. Adventitial Cells. The regenerative applications of ACs have primarily been focused on cardiovascular diseases thus far [63]. As described above, ACs actively engage in not only the physiological maintenance but also the pathological remodeling of blood vessels [64]. Consequently, harnessing the restorative power of ACs is key to the success of treating vascular diseases such as atherosclerosis and restenosis of vascular grafts [44, 65].

ACs (CD34+/31−) localized around adventitial vasa vasorum of the human saphenous vein (i.e., adventitial pericytes) have been shown to express typical MSC and certain PC antigens and displayed clonogenic and multilineage differentiation capacities, similar to ACs derived from other tissues [19]. ACs promoted the formation and stabilization of microvessel-like structures *in vitro*, likely through the reciprocal AC-EC interactions and paracrine cross-talk that can be inhibited by Tie-2 or PDGF-BB blockade [19]. Intramuscular injections of ACs in an immunodeficient mouse model of hindlimb ischemia revealed a significant angiogenic effect and facilitated near-full recovery of blood flow by as early as 7 days after injection [19]. ACs remained detectable after 14 days, interacting with host ECs through N-cadherin. These results indicate the potential of ACs in therapeutic angiogenesis/vasculogenesis, especially for the treatment of ischemic diseases. Lately, ACs have been shown to exhibit higher resistance to oxidative stress than ECs due to increased expression of antioxidant enzymes including catalase and superoxide dismutases (SODs) [66]. Silencing the extracellular, soluble isoform of superoxide dismutase (SOD3) in ACs resulted in the negation of their therapeutic benefit on blood flow recovery and neovascularization in a mouse model of peripheral ischemia, suggesting the involvement of SOD3 released by ACs in ischemic protection and/or vascular healing [66].

5.3. Cardiac Regeneration

5.3.1. Myogenic Endothelial Cells. The therapeutic potential of MECs in ischemic heart disease was investigated in an immunodeficient mouse model of acute myocardial infarction (AMI) [54]. Myocardial infarction (MI) was first induced by ligating the anterior descending branch of the left coronary artery. Cultured MECs, MCs, and ECs were intramyocardially injected into the ischemic myocardium immediately after the induction of MI [54]. When compared with injections of MCs and ECs, a significant improvement in cardiac contractility was recorded by echocardiography after MEC treatment [54]. Transplanted MECs attenuated ventricular fibrosis, enhanced proliferation and survival of host cardiomyocytes, and promoted local angiogenesis more effectively than MCs and ECs [54]. Despite the robust engraftment of MECs within the infarcted myocardium, only a few differentiated/transdifferentiated into cardiomyocytes [54]. Consequently the functional recovery of MEC-injected hearts resulted primarily from the greater paracrine secretion of trophic factors, especially vascular endothelial growth factor (VEGF), by MECs under hypoxia.

5.3.2. Pericytes. The therapeutic potency of PCs in cardiac regeneration has been investigated. Human muscle-derived PCs were injected into the ischemic myocardium immediately after the induction of MI in a SCID/NOD mouse model [50]. Echocardiography revealed that PC treatment attenuated left ventricular dilatation and significantly

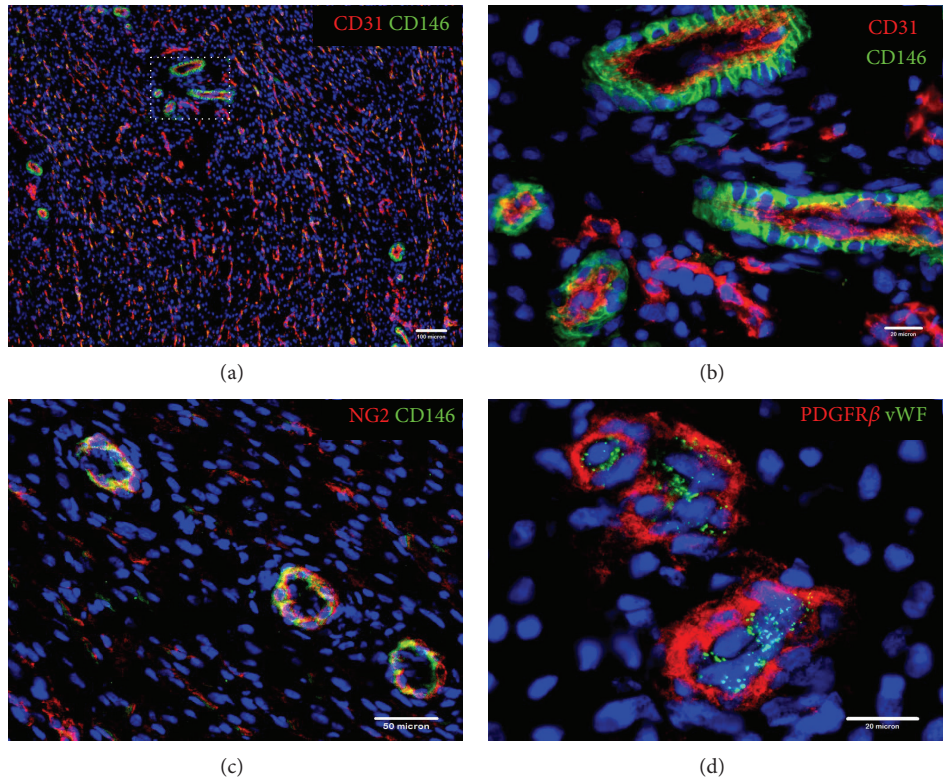

FIGURE 3: Resident microvascular pericytes in human myocardium. Human myocardium is highly vascularized with numerous microvessels of various sizes and capillaries. Resident heart microvascular pericytes can be identified by a combination of positive (pericyte) and negative (vascular endothelial) cell surface markers. (a) Microvascular and capillary endothelial cells (ECs) were stained by CD31 (red) whilst CD146+ perivascular stromal cells (green) encircled CD31+ ECs (scale bar = 100 μm). (b) Enlargement of the dotted area in (a) further showed that CD146+ human heart pericytes (hHPs, green) closely surround CD31+ ECs (red) (scale bar = 20 μm). (c) CD146+ hHPs coexpressed pericyte marker NG2 (scale bar = 50 μm). (d) hHPs expressing pericyte marker PDGFRβ (red) encircled vWF+ ECs (green) (scale bar = 20 μm). Nuclei are stained in blue by DAPI (4′,6-diamidino-2-phenylindole).

improved cardiac contractility, superior to CD56+ MCs [50]. The functional recovery was presumably attributable to the significant increase of host angiogenesis and substantial reduction of ventricular remodeling, myocardial fibrosis, and chronic inflammation at the infarct site [50]. In particular, PCs were shown to have highly active paracrine secretion of trophic factors and cytokines including VEGF-A, PDGF-β, TGF-β1, IL-6, LIF, COX-2, and HMOX-1, even under hypoxia [50]. In addition to their paracrine function, direct cellular involvement of PCs in cardiac repair was demonstrated by PC homing to perivascular locations and PC-EC interaction *in vitro* and *in vivo* as well as a fraction of PCs differentiating into and/or fusing with cardiac cells [50]. These data suggest that benefits of intramyocardial transplantation of PCs can be attributed to multiple restorative mechanisms involving paracrine effect, cellular interaction, and direct differentiation.

Very recently, we have successfully identified human heart pericytes (hHPs) based on their surface antigen expression (Figure 3) and purified hHPs by FACS from myocardial biopsies [48]. hHPs (CD146+/CD34−/CD45−/CD56−/CD117−) shared many similarities with their skeletal muscle-derived counterparts and yet showed distinctive antigenic, myogenic, and angiogenic characteristics [48]. Cultured hHPs exhibited typical mesodermal multipotency, except skeletal myogenesis, and displayed prevailing angiogenic reactions under hypoxic conditions when compared with isogenic muscle-derived PCs [48]. Our results suggest developmental and functional divergence of PCs due to anatomical specification. Interestingly, two subpopulations of PCs (Nestin−/NG2+, type-1, and Nestin+/NG2+, type-2) with differential developmental capacities have also been identified within the skeletal muscle [67]. This further suggests that the heterogeneity and developmental divergence of PCs exist not only between PCs from different organs but also among PCs within the same tissue. The tissue-specific signaling and therapeutic potential of hHPs in cardiac regeneration are currently under investigation. Collectively these data suggest that PCs serve as potent regenerative units and growth factor/cytokine sources during tissue regeneration and represent a promising stem cell reservoir, readily accessible throughout the human body, for various therapeutic applications [68].

5.3.3. Adventitial Cells. Katare et al. investigated the efficacy of ACs for treating ischemic heart disease in a mouse MI model [52]. AC treatment improved overall cardiac function and excelled BM-MSC treatment in terms of ameliorating left ventricular dilatation and wall thinning [52].

AC treatment increased local neovascularization, improved myocardial blood flow, and attenuated myocardial fibrosis, cardiomyocyte apoptosis, and vascular permeability [52]. Mechanistically, the paracrine secretion of proangiogenic factors and chemokines presumably activated the proangiogenic and prosurvival Akt/eNOS/Bcl-2 signaling pathway. The involvement of microRNA-132 (miR-132) as a novel paracrine angiogenic stimulant and remodeling inhibitor was demonstrated by blocking miR-132 function in ACs using anti-miR-132, which in turn significantly decreased their vascular supportive capacity *in vitro*, revascularization in the ischemic myocardium, and cardiac reparative/protective functions [52]. Very recently, a combinatory therapy with c-kit+ cardiac stem cells and adventitial pericytes derived from human saphenous vein has been shown to exhibit additive benefits for cardiac regeneration [69]. Together these data strongly support the therapeutic value of ACs in cardiovascular diseases.

5.4. Skin Regeneration

5.4.1. Pericytes. The presence of mesenchymal cells, presumably taking on the role of PCs in the healing wound, was first observed in 1970 [70]. The involvement of multipotent dermal PCs in epidermal tissue renewal has been supported by the significantly enhanced regenerative capacity of committed human epidermal cells in organotypic coculture, presumably through the augmented secretion of laminin-α5 and independent of angiogenesis, suggesting the importance of pericyte-mediated remodeling of local ECM microenvironment [71]. The role of PCs in wound healing and cell-based wound therapy was reviewed in [35].

5.5. Bone Regeneration

5.5.1. Myogenic Endothelial Cells. The potential of MECs for bone regeneration has been examined *in vitro* and in mice [28, 49]. MECs exhibited intense mineralization in pellet culture only with the presence of bone morphogenetic protein- (BMP-) 4, suggesting no spontaneous osteogenic differentiation of MECs without an appropriate inductive signal [49]. μCT imaging revealed that MECs transduced with BMP-4 formed dense ectopic bone nodules when seeded onto a gelatin sponge and implanted into intramuscular pockets in immunodeficient mice [49]. Currently the potency of MEC transplantation for the treatment of critical bone defects is under investigation.

5.5.2. Pericytes. Together with ACs, human adipose-derived PCs have been extensively studied for their bone regenerative capacity [47, 72]. Interestingly, although most demographic parameters, including age, gender, and menopause, did not affect adipose PC yield, donors with body mass index (BMI) less than 25 (nonoverweight) appeared to have higher PC yield than obese donors (BMI > 30) [47]. Moreover, human umbilical cord CD146+ perivascular cells have also been demonstrated as a promising cell source for bone regeneration [73].

5.5.3. Adventitial Cells. Combined with PCs, the skeletal regenerative capacity of human adipose-derived ACs has been explored [47, 72]. Human perivascular stem cells (hPSCs), which comprised only PCs and ACs, were purified from lipoaspirate SVF, seeded onto osteoinductive or control collagen scaffolds, and implanted into either intramuscular ectopic implantation model or critical-sized calvarial bone injury model in immunodeficient mice [47, 72]. When compared with unfractionated SVF, hPSCs formed significantly more bone intramuscularly and led to dramatically greater healing of critical-sized calvarial defects [47, 72]. Additionally, unlike BMP-2 which increased bone formation by hPSCs *in vivo* but also induced an adipogenic response, Nel-like molecule 1 (NELL-1) selectively enhanced osteogenesis of hPSCs and therefore represents a novel osteoinductive growth factor for hPSC-mediated skeletal regeneration [72]. Together recent studies suggest that ACs, like PCs, are functionally superior MSC alternatives for regenerative purposes and effortlessly accessible from dispensable tissues such as lipoaspirate.

6. Conclusion

The capability to isolate subpopulations of hBVSCs marked a major progress to understand the heterogeneous MSC entity as well as their vascular/perivascular niches. Purified MECs, PCs, and ACs exhibited robust reparative/regenerative capacities in many injured/defective tissues, often outperforming unfractionated MSCs. No tumorigenesis of any hBVSC subset has been reported thus far, indicating their safety for translational applications. More preclinical studies with large animal models are necessary to further validate the therapeutic safety and efficacy of hBVSCs for clinical use. Currently researchers have planned clinical trials using human pericytes in patients with refractory myocardial ischemia [74]. In addition to fresh tissue biopsies, we have been able to purify MECs and PCs from long-term cryopreserved human primary skeletal muscle cell cultures and further demonstrated their sustained myogenic capacity *in vivo* [75]. This suggests the feasibility to purify specific subset(s) of hBVSCs from either fresh biopsy or banked human primary cells, ultimately facilitating customized regenerative medicine using personalized, homogeneous therapeutic stem/progenitor cell population(s) for a particular pathological condition.

Conflict of Interests

Johnny Huard received remuneration from Cook MyoSite Inc. for consulting services and for royalties received from technology licensing during the period in which the above research was performed. All other authors have no conflict of interests to disclose.

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