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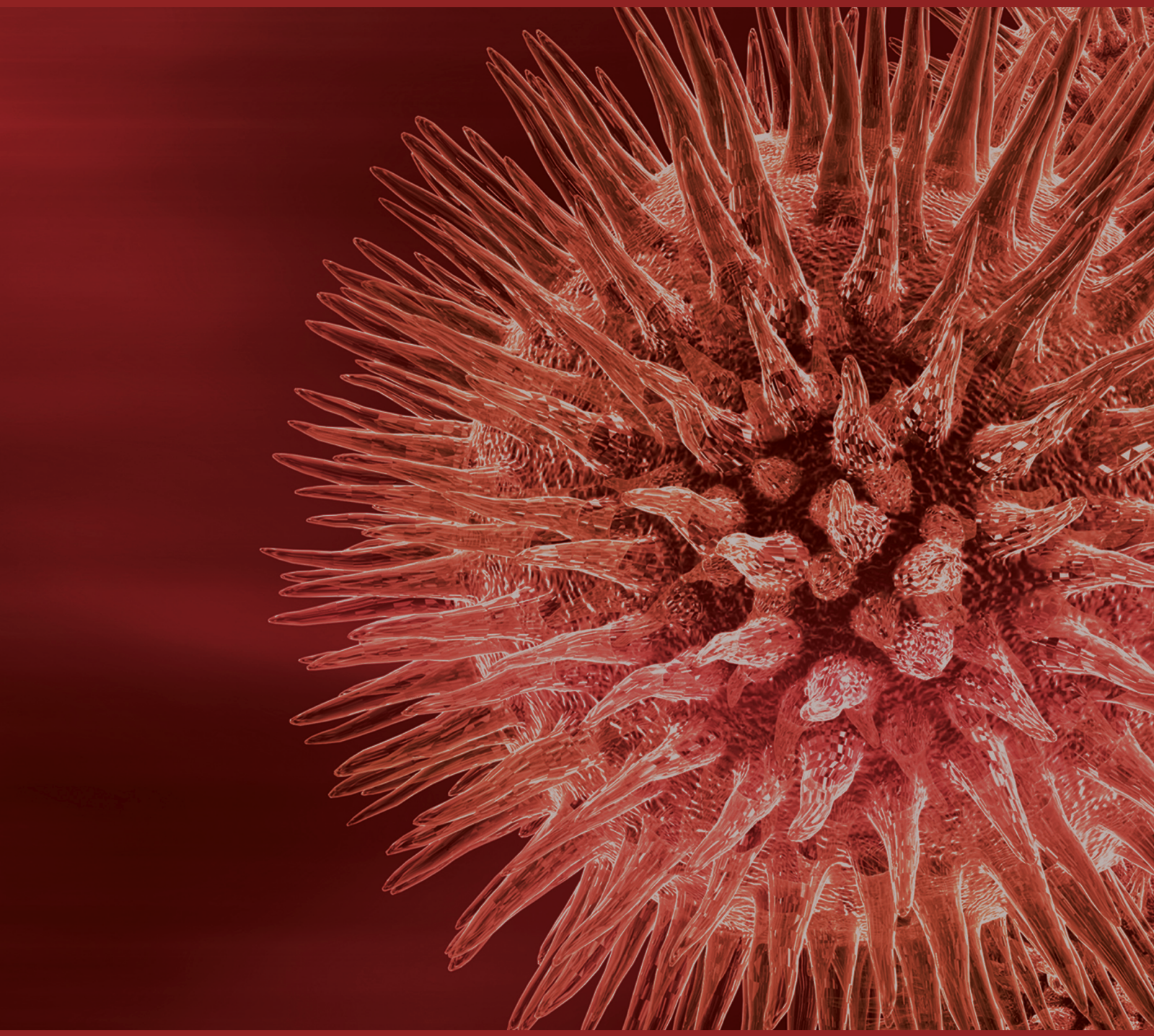
Toward Personalized Cell Therapies by Using Stem Cells 2012

Guest Editors: Ken-ichi Isobe, Herman S. Cheung, and Ji Wu



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Editorial

Toward Personalized Cell Therapies by Using Stem Cells 2012

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The iPSCs technology opens the possibility of personalized cell therapies for treating human disease and/or repairing the damaged tissues. Multipotent adult stem/progenitor cells including mesenchymal stem cells (MSCs) also have the potential to use personalized cell therapy.

One target of stem cell therapy is to apply genetic disorders, which are difficult to treat by other medical ways. Q. Jiang et al. showed interesting experiment by using MSC. They transfected Abcc6 gene into MSC and differentiated to hepatic cells and transplanted Pseudoxanthoma Elasticum model mouse.

Myocardial infarction and ensuing heart failure are the leading causes of mortality in western countries. "Cardiospheres" are self-assembling spherical clusters of cells obtained by outgrowth from cardiac explants in the primary *ex vivo* tissue culture. CSs placed in a new culture dish disassembled and gave rise to a monolayer of CS-derived cells (CDCs) that formed second-generation CSs. CDCs could be expanded as monolayers on fibronectin. L. Barile et al. introduced the first clinical trial of autologous c-kit+/Lin− CSCs for treatment of heart failure which improved left ventricular function of ischemic heart disease.

One of the most important applications of stem cell therapy is end-stage renal disease (ESRD). More than 290,000 ESRD patients are currently undergoing dialysis in Japan. S. Yokota et al. reviewed recent works, which target the stem cell therapies to ESRD.

K. Chenard et al. have summarized current clinical therapy of craniofacial reconstruction by BMPs; they proposed future personalized therapy by culturing MSCs with BMPs.

In this second edition, we found the paper, which discuss or examine the controversial issues for achieving cell therapy safely and effectively. P. Nascimento reviewed the problems to be analyzed before practical use of stem cells: methods for stem cells culture, teratogenic or tumorigenic potential, cellular dose, proliferation, senescence, karyotyping, and immunosuppressive activity. Recently, it has been shown that MSCs have immunoregulatory functions. E. Ivanova-Todorova et al. reported that MSCs lead to increased expression of FoxP3 regulatory T cells.

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Ken-ichi Isobe
Herman S. Cheung
Ji Wu

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Research Article

Administration of Bone Marrow Derived Mesenchymal Stem Cells into the Liver: Potential to Rescue Pseudoxanthoma Elasticum in a Mouse Model (*Abcc6*$^{-/-}$)

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Pseudoxanthoma elasticum (PXE) is a heritable ectopic mineralization disorder caused by loss-of-function mutations in the *ABCC6* gene which is primarily expressed in the liver. There is currently no effective treatment for PXE. In this study, we characterized bone marrow derived mesenchymal stem cells (MSCs) and evaluated their ability to contribute to liver regeneration, with the aim to rescue PXE phenotype. The MSCs, isolated from GFP-transgenic mice by magnetic cell sorting, were shown to have high potential for hepatic differentiation, with expression of *Abcc6*, in culture. These cells were transplanted into the livers of 4-week-old immunodeficient *Abcc6*$^{-/-}$ mice by intrasplenic injection one day after partial hepatectomy, when peak expression of the stromal cell derived factor-1 (SDF-1) in the liver was observed. Fluorescent bioimaging analyses indicated that transplanted MSCs homed into liver between day 1 and 7, and significant numbers of GFP-positive cells were confirmed in the liver by immunofluorescence. Moreover, enhanced engraftment efficiency was observed with MSCs with high expression levels of the chemokine receptor Cxcr4, a receptor for SDF-1. These data suggest that purified MSCs have the capability of differentiating into hepatic lineages relevant to PXE pathogenesis and may contribute to partial correction of the PXE phenotype.

1. Introduction

Pseudoxanthoma elasticum (PXE), a life-altering and frequently devastating disease, affects the skin, the eyes, and the cardiovascular system with ectopic mineralization [1, 2]. PXE is caused by the mutations in the *ABCC6* gene, which encodes a member of the C-family of ATP-binding cassette transporters [3, 4]. Surprisingly, this gene appears to be expressed primarily in the liver and the kidneys, tissues not clinically affected in PXE [5]. We have developed an *Abcc6*$^{-/-}$ mouse model by targeted ablation of the *Abcc6* gene [6]. These mice recapitulate histopathologic features of human PXE, and serve as an excellent model system to study pathomechanisms leading to tissue mineralization as a result of *Abcc6* inactivation, and they serve as a platform to evaluate the curative effects of different treatment modalities. Recently, we have demonstrated that PXE is a metabolic disorder with the primary pathology in the liver and with secondary involvement of elastic fibers in soft tissues [7, 8]. However, the precise function of *ABCC6* protein, consequences of the *ABCC6* mutations at the mRNA and protein levels, and the pathomechanisms leading to mineralization of the elastic fiber structures are largely unknown. Currently, there are no treatment modalities available for this disorder.

Various therapeutic strategies have been explored in clinical trials based on cutting-edge basic research on liver metabolic diseases. Gene therapy and cellular therapy are overlapping fields of biomedical research with similar therapeutic goals of tissue regeneration. However, relatively little progress has been made in gene therapy since the first clinical trial in 1990 [9]. Short-lived nature of gene therapy and problems of safety with viral vectors have kept gene therapy from becoming an effective treatment for many genetic diseases [10, 11]. Liver transplantation might be an effective therapy for severe liver diseases, but few patients can benefit from this procedure due to the shortage of donor organs.

Moreover, the whole organ transplantation involves major surgery, is highly invasive and requires lifelong immunosuppression. Currently, cellular therapy with stem cells and their progeny is a promising new approach capable of addressing mostly unmet medical needs. The considerable excitement surrounding the stem cell field is based on the unique biological properties of these cells and their capacity to self-renew and regenerate tissue and organ systems. Specifically, bone marrow stromal cells are an attractive source for cell-based gene therapy to genetic liver disorders, and their capability of differentiating into hepatocyte lineage has been demonstrated previously [12, 13]. The cell transplantation has been performed in several patients with modest liver metabolic correction, such as in the patients with Crigler-Najjar syndrome and with advanced liver failure [14–16]. It appears, therefore, that PXE would be an appropriate candidate disease to test cell-based therapeutics.

Hereby, we preliminarily evaluated a stem-cell-based therapeutic approach for PXE by assessment of the potential of MSCs in liver reconstitution with the aim to rescue the PXE phenotype in *Abcc6*$^{-/-}$ mice and eventually on patients.

2. Materials and Methods

2.1. Mice and Cell Transplantation. An immunodeficient PXE mouse model [8], generated by crossbreeding the traditional *Abcc6*$^{-/-}$ mouse [6] with a well-established immunodeficient *Rag1*$^{-/-}$ mouse in C57BL/6 background (strain: 002216F; The Jackson Laboratory, Bar Harbor, ME), was used in this study. four-week-old mice were anesthetized and 50% partial hepatectomy (PHx) was performed following the standard protocol of Higgins and Anderson [17]. For the administration of cells, approximately 5×10^5 Cxcr4-MSCs or unmodified MSCs (see below) at passage 6, isolated from GFP-transgenic mice (C57BL/6-Tg UBC-GFP; The Jackson Laboratory) as the source of donor cells, were delivered into the recipient mouse liver by intrasplenic injection at 24 hours after PHx. The mice were maintained under pathogen-free conditions and were handled in accordance with the guidelines for animal experiments by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

2.2. Bone Marrow Derived Stem Cell Isolation and Characterization. The method of magnetic cell sorting (MACS) was utilized to obtain desired populations of bone marrow derived mesenchymal stem cells (MSCs) as described by manufacturer (Miltenyi Biotec, Cambridge, MA). Briefly, wild-type mouse MSCs were harvested from 4-week-old GFP-transgenic mice and enriched by immunomagnetic separation strategies using cocktails of antibodies that deplete the differentiated cells of hematolymphoid lineages (Lin), such as the cells expressing the following lineage antigens: CD5, CD45R, CD11b, Gr-1, 7-4, and Ter-119. To obtain a pure population of stem cells, positive selection with Sca-1 antibody was utilized to further sort cells by MACS. These cells were maintained in the MSC medium containing MesenCult MSC Basal Medium (Stemcell Technologies, Vancouver, Canada), 20% Mesenchymal Stem Cell Stimulatory Supplements (Stemcell Technologies), 100 unit/mL penicillin (Invitrogen, Carlsbad, CA), 100 μg/mL streptomycin (Invitrogen), and 2.5 μg/mL amphotericin B (Invitrogen). The culture medium was changed every 3 days. Mouse MSCs at passage 6, identified as Lin$^-$CD45$^-$CD31$^-$Sca-1$^+$, were used as MSCs for the experiments.

To characterize the cell surface markers by flow cytometric analysis, harvested MSCs were incubated at 4°C for 30 minutes with rat anti-mouse CD11b, CD45, CD105, CD106, Sca-1, CD29, or MHC-1 antibody (R&D system, Minneapolis, MN), followed by 30 minutes incubation with APC-labeled rabbit anti-rat IgG antibody (BD Biosciences, San Jose, CA). Cells were examined by using FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed with Flowjo software (Tree Star Inc., Ashland, OR).

2.3. Transfection and Selection of MSCs Expressing Exogenous Cxcr4. MSCs at approximately 80% confluency were transfected with pCMV6-Kan/Neo carrying a full length cDNA of mouse Cxcr4 (Origene, Rockville, MD) using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were maintained in the MSC medium and subjected to the selection by G418 at the dose of 1,500 μg/mL, and switched to the maintenance dose of 500 μg/mL in 2 weeks. The positive transfected cells were described as Cxcr4-MSCs in subsequent experiments.

2.4. In Vitro Hepatic Differentiation. Prior to starting the hepatic differentiation, MSCs at passage 5 were maintained in the regular MSC culture medium until at 80–90% confluence. The hepatic differentiation was elicited in the differentiation-inducing medium, which consisted of DMEM (Invitrogen) supplemented with 10% FBS, 10 ng/mL hepatocyte growth factor (HGF) (PeproTech Inc, Rocky Hill, NJ), 10 ng/mL basic fibroblast growth factor (bFGF) (PeproTech INC) and 10 ng/mL oncostatin M (R&D system). The medium was changed every 3 days, and the cells were cultured for 8 days.

2.5. Immunofluoresence. To analyze the protein expression in the differentiated MSCs, the cells either in the regular MSC culture medium or in the differentiation-inducing medium were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 at day 8. The cells were incubated with mouse anti-human mouse albumin (Alb) antibody (R&D system) or rabbit anti-mouse cytokeratin (CK)-18 antibody (Novus, Littleton, CO), followed by the incubation with the second antibody, Texas Red conjugated anti-rabbit IgG (Molecular Probe, Eugene, Oregon). DAPI was used for nuclear counterstaining.

To examine the presence of GFP positive transplanted MSCs in the liver, the engrafted livers were removed, fixed with 4% paraformaldehyde, processed in a gradient sucrose, and then subjected to immunofluorescent analysis. The processed livers were embedded in Tissue-tec OCT Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and stored at −80°C until use. Six-μm-thick frozen sections of the liver were incubated with rabbit anti-GFP antibody (Invitrogen).

Subsequently, sections were stained with FITC goat anti rabbit IgG secondary antibody (Invitrogen).

2.6. RT-PCR and qPCR. To examine the liver specific gene expression in differentiated MSCs, total RNA was extracted from the cultured regular MSCs or the differentiated MSCs at day 8 of culture using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples were subjected to random-primed reverse transcription by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). RT reaction products were used for PCR for amplifying mouse CK-18, hepatocyte nuclear factor (HNF)-3b and *Abcc6*, with actin as an internal control. To analyze the expression of Cxcr4 in the transfected cells with pCMV6-Cxcr4 plasmid, RNA isolation and reverse transcription procedures were performed as above with on-column DNA digestion. The PCR was performed to amplify mouse *Cxcr4* gene in transfected or untransfected cells with Gapdh as an internal control.

To quantitate the percentage of migrated GFP positive MSCs into the liver by qPCR, total DNA was extracted from homogenized engrafted liver using the DNeasy Kit (Qiagen). SYBR Green PCR amplification of GFP was performed in ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBER Green PCR Master Mix (Applied Biosystems). The amount of GFP^+ DNA in each 50 ng DNA sample was quantified and normalized to interleukin (IL)-2 DNA. The relative expression level of the target gene was calculated using the ΔΔCt method. To prepare the standard curve, genomic DNA from the wild type mouse was spiked with serial dilutions of genomic DNA from the GFP-transgenic mouse. The diluted GFP standard samples were subjected to the PCR amplification of GFP and IL-2, and the percentage of GFP transgene per 50 ng tissue-derived DNA was calculated.

2.7. ELISA for Murine SDF-1. Serum samples were collected from the mice before PHx, and at 2 hours, 1 day, 3 days, and 7 days after the surgery. SDF-1 levels of mouse serum were determined using the mouse SDF-1 ELISA Kit (R&D system) according to the manufacturer's instructions. Liver samples were harvested from mice at the designated time points after the surgery and homogenized using a homogenizer and a 27 gauge needle in the RIPA buffer (Sigma-Aldrich, St. Louis, MO) supplemented with the proteinase inhibitor cocktail (Complete, Mini; Roche Applied Science, Indianapolis, IN) at 4°C. The mixture was centrifuged for 20 minutes at 12,000 rpm at 4°C and the supernatant was collected. The SDF-1 concentration in the liver extracts was examined by using the mouse SDF-1 ELISA Kit (R&D system) and normalized by the total protein concentration measured by BCA Protein Assay Kit (Pierce, Rockford, IL).

2.8. Migration Determination In Vitro and In Vivo. In vitro migration assays were carried out in a 48-well transwell using polycarbonate membranes with 8-μm pores (Osmonics; Livermore, CA). After different concentrations of mouse recombinant SDF-1 (R&D system) were added to the lower chamber, 2.5×10^3 MSCs or Cxcr4-MSCs in 30 μL of DMEM with 0.5% FBS were placed in the upper chamber of the transwell assembly. After incubation at 37°C and 5% CO_2 for 4 hours, the upper surface of the membrane was scraped gently to remove nonmigrating cells and washed with PBS. The cells on the membrane were fixed in 4% paraformaldehyde for 15 minutes and stained with Giemsa. The number of migrating cells was determined by counting 3 random fields per well under the microscope at 100x magnification. Experiments were performed in triplicate.

To assess the *in vivo* migration ability of MSCs, fluorescence bio-imaging system was utilized. The mice transplanted with MSCs labeled with a fluorescent dye, Vybrant DiD (Molecular Probes, Eugene, OR) were monitored at multiple time points (day 1, week 1, week 2, and week 3) by using an IVIS Luminar XR live imaging system (Caliper, Hopkinton, MA) at excitation filter 644 nm and emission filter 665 nm.

2.9. Data and Statistical Analysis. Statistical analyses were performed with Student's *t*-test. *P* values <0.05 were considered statistically significant.

3. Results and Discussion

3.1. MSC Isolation and Cell-Surface Antigen Profile. Total bone marrow from GFP-transgenic mice was obtained by purging the medullar canal of the femurs and tibias with a 26 gauge needle, and MSCs were purified by magnetic cell sorting using antibodies against a panel of hematolymphoid lineage antigens for negative selection and then antibodies against Sca-1 for positive selection (see Section 2). Flow cytometric analysis of freshly isolated cells demonstrated that approximately 99% cell populations were both GFP and Sca-1 positive (data not shown). The cells were cultured in the regular MSC medium and passaged when they reached 80% confluence. Under the microscopic observation, the cells at early passage (passage 3) exhibited a thin spindle like shape and became large fibroblast-like in a swirl pattern at passage 5 (Figure 1(a)). Flow cytometric analyses showed a positive histogram peak for Sca-1 and CD29, but both positive and negative histogram peaks for CD11b, CD45, and CD106 were observed with the cells at passage 3. The histogram positive peak for CD11b and CD45, and negative peak for CD106 disappeared at passage 5 (Figure 1(b)). These observations indicated the presence of two populations: hematopoietic stem cells (HSCs) and MSCs, and that a significant enrichment in MSCs was gained during subsequent culturing of the cells. MSCs have been shown to be a resource of hepatic differentiation, but there are no specific markers of MSCs. Thus, to obtain purified population of MSCs, a further purification may be needed by *in vitro* culture following selection using by a group of surface markers.

The cells at passage 3, 5, or 7 were also analyzed by FACS for their immunologic properties using monoclonal antibody to mouse MHC class I. MSCs (passage 3, 5, and 7) derived from the same mouse expressed high levels of MHC class I when compared to melanoma derived cells (BL-TAC, negative control) and mouse fibrosarcoma derived cells (MC57G, positive control) (Figure 2). In the literature, it has

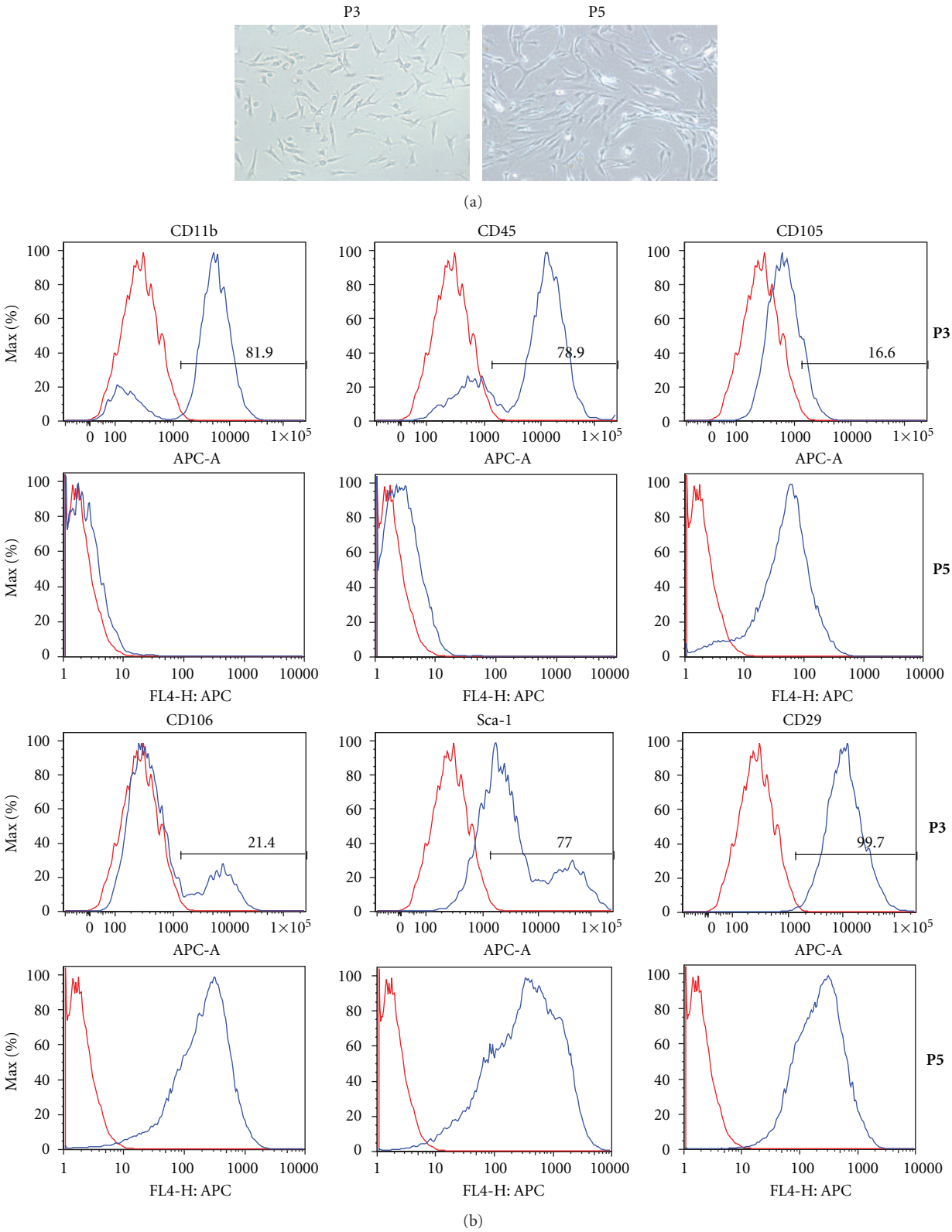

Figure 1: Characterization of the mouse bone marrow derived stem cells. The bone marrow derived stem cells were isolated by magnetic cell sorting using a Lin^- cocktail of antibodies as well as stem cell antigen-1 (Sca-1) antibody and then cultured in the regular stem cell culture medium. (a) The cells were observed by phase contrast microscopy at passage 3 (left) or passage 5 (right). The cells at passage 5 became more fibroblastic appearing compared to passage 3. (b) Flow cytometry was utilized to examine the surface markers of cells at passage 3 (top panels) or passage 5 (bottom panels) for the purity of cell population.

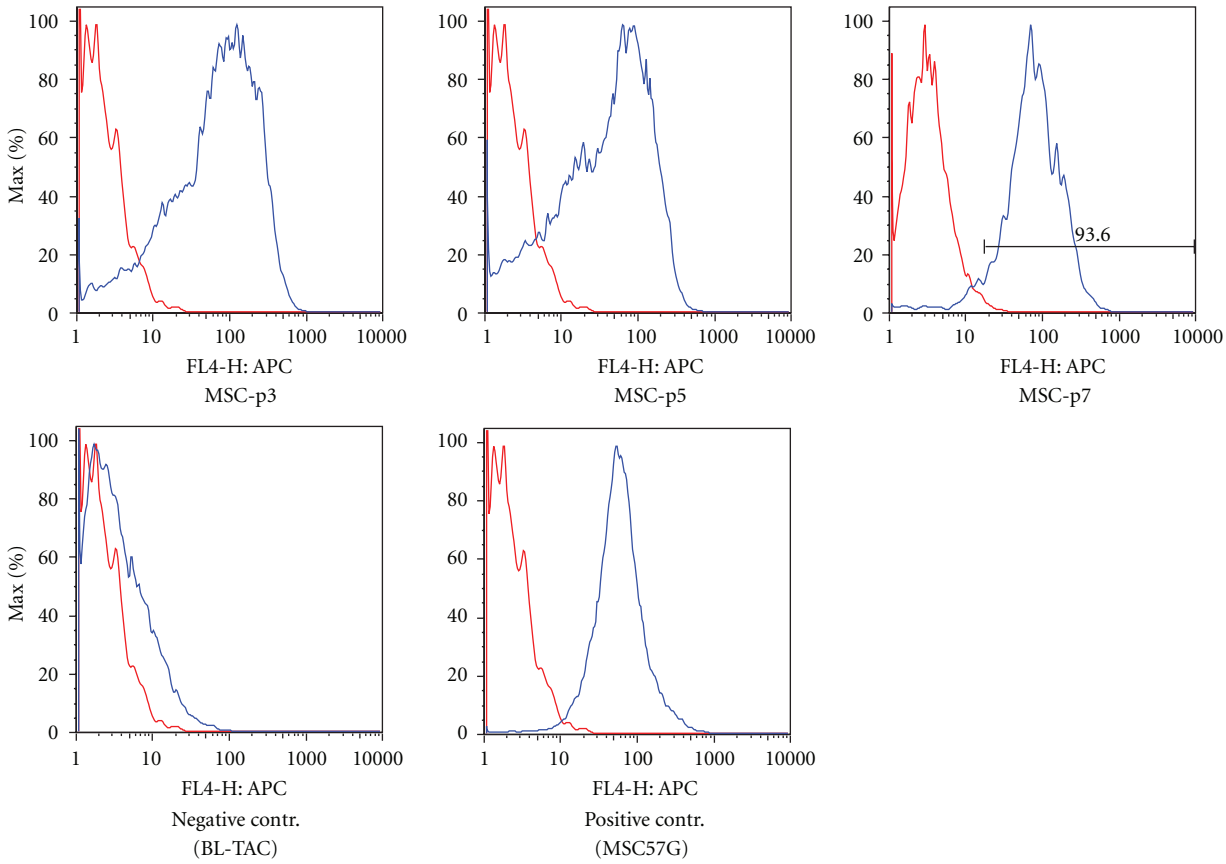

Figure 2: Flow cytometric characterization of MHC class I expression in bone marrow derived stem cells. The cells at passage 3 (MSC-p3), 5 (MSC-p5) or 7 (MSC-p7) were analyzed by FACS using monoclonal antibody to mouse MHC class I. Mouse melanoma derived cells (BL-TAC) and mouse fibrosarcoma derived cells (MSC57G) were used as negative and positive controls, respectively.

been suggested that MSCs may be immunoprivileged and can be transplantable between MHC-incompatible individuals [18]. On the other hand, it has been suggested that MSCs are not intrinsically immunoprivileged and could not serve as a "universal donor" in immunocompetent MHC-mismatched recipients [19]. In our study, high expression of MHC class I supported the latter possibility and suggested that modulation of immune response may be of concern when these cells are employed *in vivo*.

3.2. Differentiation Potential of Bone Marrow Stem Cells into Hepatocytes In Vitro. To investigate the potential of hepatic differentiation of MSCs, the purified MSCs at passage 6 were cultured for 8 days in differentiation-inducing medium, that is, DMEM containing 10% fetal bovine serum, 10 ng/mL HGF, 10 ng/mL bFGF, and 10 ng/mL oncostatin M. The cell morphology changed as they became extended and larger (Figure 3(a), top panel). Immunofluorescence analysis was performed for the expression of the hepatocyte-specific proteins and demonstrated that CK-18 and Alb were positively expressed in the differentiated cells but not in the undifferentiated ones (Figure 3(a), middle and bottom panels). HNFs and CK-18, key players for the hepatogenesis, were expressed in the differentiated cells by RT-PCR, while negative in control cells cultured with the MSC culture medium for the same period (Figure 3(b)). MSCs, maintained either in differentiation-inducing medium or under basal condition, expressed *Abcc6* mRNA at comparable levels.

The differentiation process of stem cells requires a specific microenvironment. The results clearly demonstrated that the isolated mouse MSCs are able to differentiate into hepatocytes under *in vitro* conditions in medium containing growth factors HGF, bFGF and oncostatin M, which have been reported to be important components for liver development and differentiation [20, 21]. In addition, our differentiated cells could show the expression of the *Abcc6* gene, which is lost in PXE and may be needed to restore its phenotype. These data provide critical information about stem cell biology that should contribute to the development of regenerative medicine for liver diseases in general and for PXE in particular.

3.3. Migration Capability of Bone Marrow Stem Cells

3.3.1. MSCs Cxcr4 Expression and In Vitro Migration. MSCs at passage 4 were transfected with a murine Cxcr4 expression vector driven by CMV promoter, pCMV6-mCxcr4, and then selected with G418 to obtain cells with stable expression (Cxcr4-MSCs). The Cxcr4-MSCs were analyzed for expression of Cxcr4 first by RT-PCR. High level of Cxcr4 mRNA

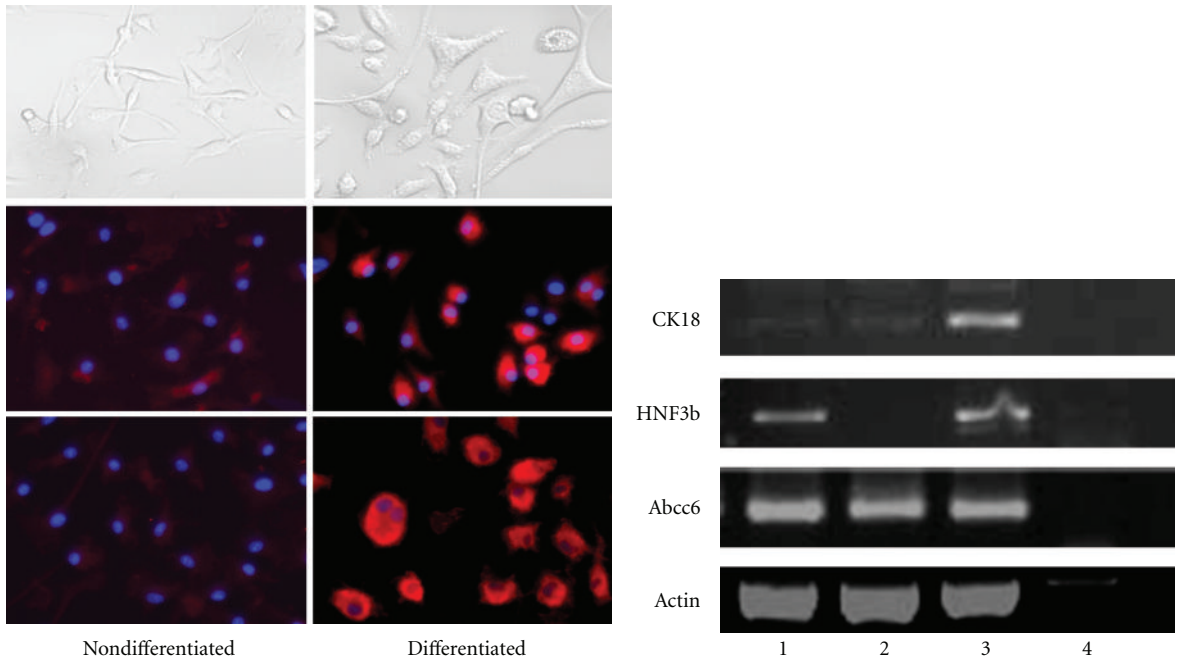

Figure 3: Hepatocytic differentiation capacity of mouse bone marrow stem cells *in vitro*. The cells at passage 5 were cultured either in the regular stem cell culture medium (a, left panels) or in the differentiation-inducing medium (a, right panels). As observed by phase contrast microscopy at day 8 (top panels), the cells became extended and larger in the differentiation-inducing medium (a, right top). Immunofluorescent analysis was performed at day 8 of differentiation. Cells were stained with antibodies to liver-specific marker proteins, albumin (a, middle panels), or CK-18 (a, bottom panels). DAPI staining was used to identify the nuclei (blue). Strong signals were observed in the cells cultured in the differentiation-inducing medium (a, right middle and bottom panels), while weak signal (a, left middle and bottom panels), if any, was detected in the cells cultured in the regular stem cell medium. (b) RT-PCR was conducted and the mRNA levels of different liver-specific genes were examined. They are cytokeratin-18 (CK-18), hepatocyte nuclear factor 3b (HNF3b) and *Abcc6*, respectively. Lane 1: cultured cells in differentiation-inducing medium; lane 2: cultured cells in stem cell culture medium; lane 3: MLE-10 (mouse liver epithelial cell line); lane 4: H_2O blank.

was observed in these cells while lower, barely detectable levels were present in untransfected MSCs (Figure 4).

To evaluate cell migration in response to SDF-1, the ligand for Cxcr4, we examined whether exogenous overexpression of Cxcr4 enhances the chemotaxis of MSCs towards an SDF-1 gradient in a transwell migration assay. Cxcr4 modification of MSCs increased the number of cells migrating towards SDF-1 in a dose-dependent pattern (Figure 5(a)). Specially, the number of migrated cells significantly increased at the dose of 30 and 60 ng/mL when compared to that without adding SDF-1 (Figure 5(b)). Few migrated cells were found in untransfected MSCs with all different dose settings of SDF-1 (data not shown). These data indicate that overexpression of Cxcr4 enhances the ability of MSCs to respond to SDF-1 induced chemotaxis.

3.3.2. The Levels of SDF-1 in the Liver and the Serum after Partial Hepatectomy. To investigate how partial hepatectomy affects the levels of SDF-1 in the liver tissue or in the blood, we first determined the temporal expression of SDF-1 mRNA in the hepatectomized livers by quantitative RT-PCR. SDF-1 increased at day 1 by more than 2-fold and then decreased gradually to reach the baseline level as before partial hepatecomy at day 7 (Figure 6(c)). Using the total

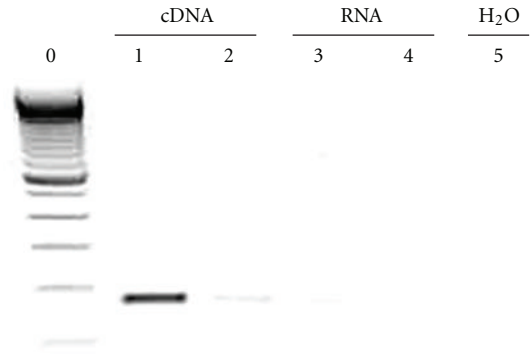

Figure 4: Overexpression of Cxcr4 in bone marrow derived stem cells. The cells at passage 5 were transfected with a mouse Cxcr4 expression vector (pCMV6-Cxcr4) and the stable cell clones expressing Cxcr4 were established. RT-PCR was conducted to examine the mRNA levels of mouse Crcx4 in the transfected or untransfected stem cells at the same passage. Lane 0: 100 bp DNA ladder; lane 1: reverse transcribed sample from the transfected cells; lane 2: reverse transcribed sample from the untransfected cells; lane 3: nonreverse transcribed sample from the transfected cells; lane 4: nonreverse transcribed sample from the untransfected cells; lane 5: H_2O blank.

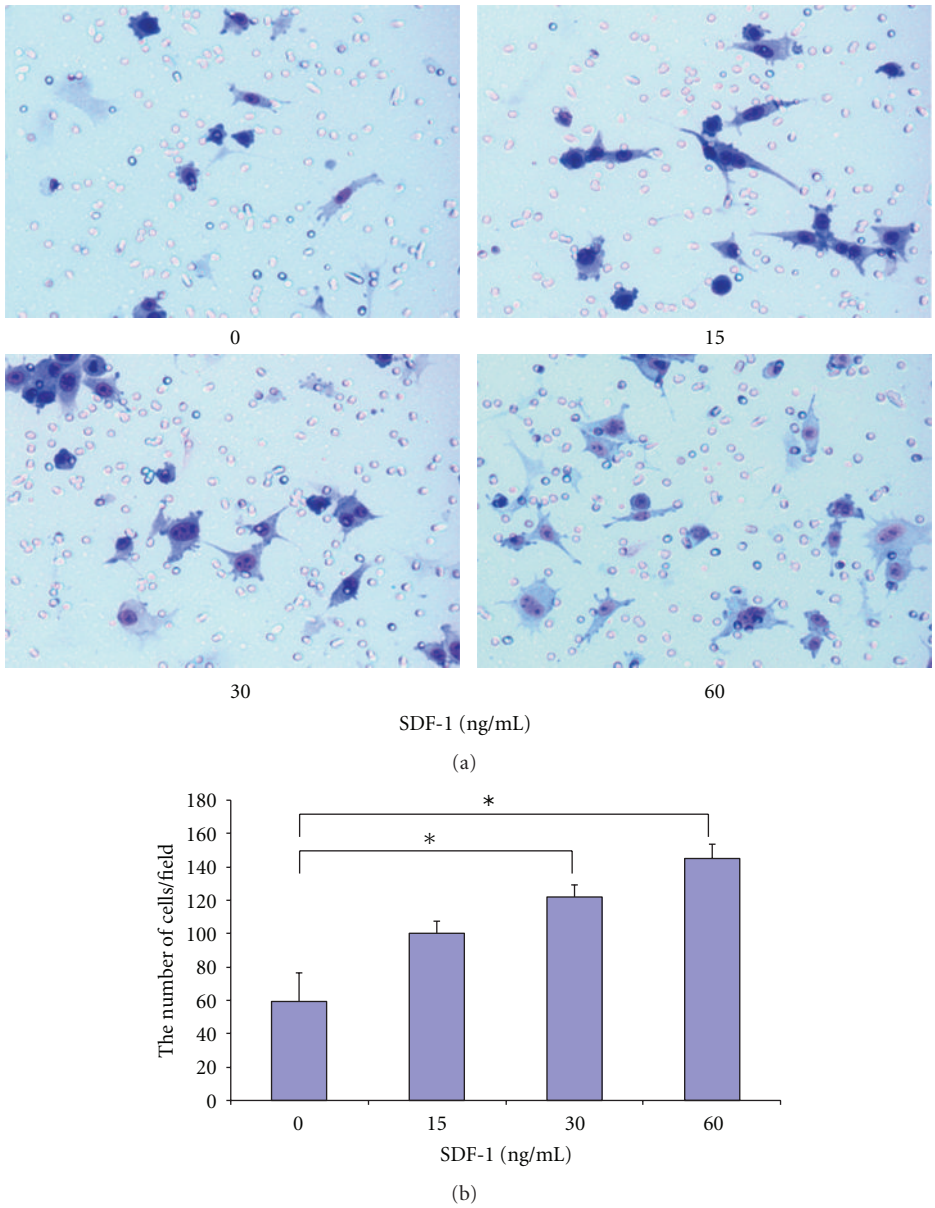

Figure 5: *In vitro* migration of Cxcr4-MSCs. (a) Representative images of transmigrated Mesenchymal stem cells (MSCs) stably overexpressing Cxcr4 in response to stromal-derived factor-1 (SDF-1) at concentrations of 0, 15, 30, or 60 ng/mL in transwell assay. (b) Average number of cells migrated in transwell migration assay counted in 100x magnification field. Results are mean ± SEM of 3 different fields from 3 independent experiments. The asterisks indicate statistically significant differences, $P < 0.05$ (Student's t-test).

liver protein extracts, ELISA showed a significant increase in the hepatectomized livers at day 1 and then the levels of SDF-1 quickly returned down to the baseline at day 3 and 7 after partial hepatectomy (Figure 6(d)), consistent with the changes at mRNA levels. Conversely, the serum levels of SDF-1 as measured by ELISA were decreased at 2 and 24 hours, and then returned to the baseline by 180 hours (day 7) (Figure 6(b)).

3.3.3. Homing of Transplanted MSCs towards the Liver. At 24 hours after partial hepatectomy in immunodeficient *Abcc6*$^{-/-}$ mouse model, *Abcc6*$^{-/-}$; *Rag1*$^{-/-}$, 5×10^5 Cxcr4-MSCs or unmodified MSCs were administrated by intrasplenic injection. The transplanted mice were monitored for DiD-labeled MSCs for their migration towards the liver by a live IVIS Luminar XR imaging system. DiD is a fluorescent tracer which allows cells to be marked in distinctive color detectable by the imaging machine. DiD labeled MSCs were detected in the liver and spleen at day 1 and day 7 after transplantation (Figure 7(a)). The presence of GFP-positive MSCs at day 7 in the liver was confirmed by immunofluoresence using anti-GFP antibody (Figure 7(b)). Moreover, to quantitate the percentage of migrated cells, genomic DNA was isolated from the harvested liver at day 7 and qPCR was performed. Using a serially diluted GFP DNA samples as the standards, the average percentage of

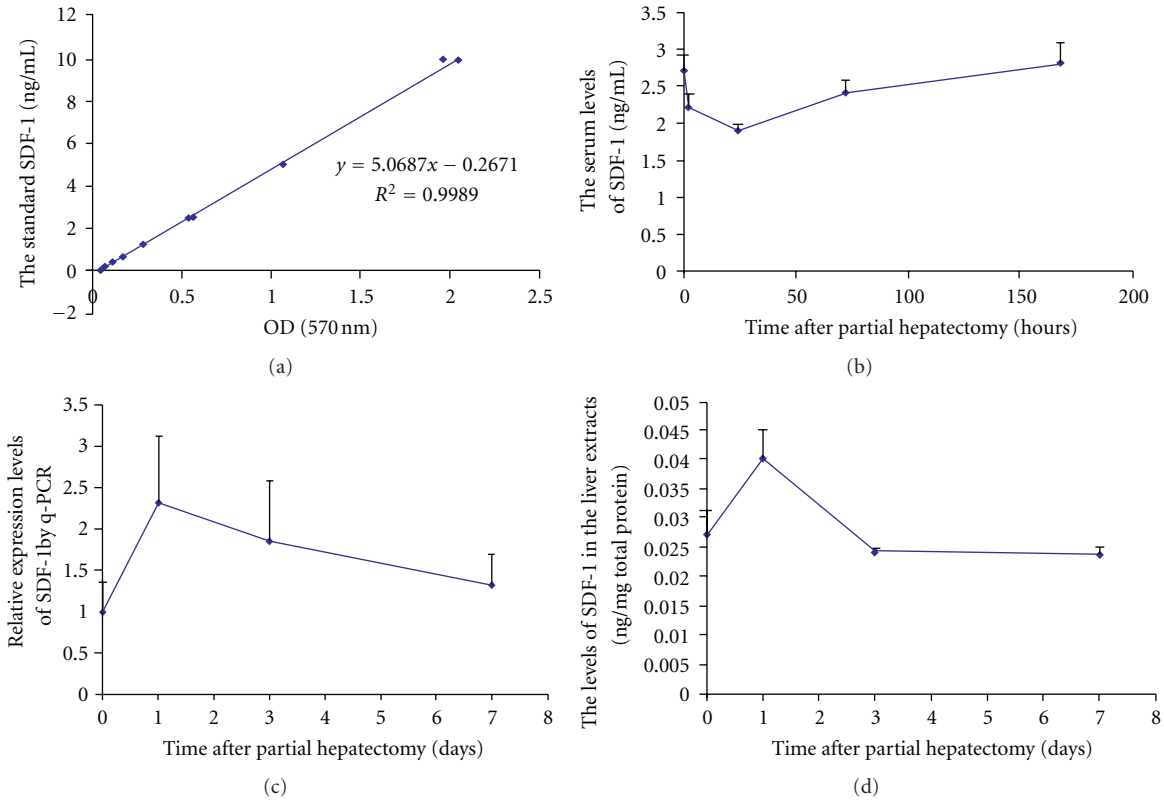

Figure 6: The levels of SDF-1 in the liver or the serum after partial hepatectomy. Serum samples (b) or liver extracts (d) were obtained from the mice at the different time points after partial hepatectomy, and analyzed to determine the levels of SDF-1 using ELISA ($n = 3$–5). The levels of SDF-1 were calculated based on the standard curve (a). RT-PCR was performed to examine the mRNA level of SDF-1 in the hepatectomized livers (c).

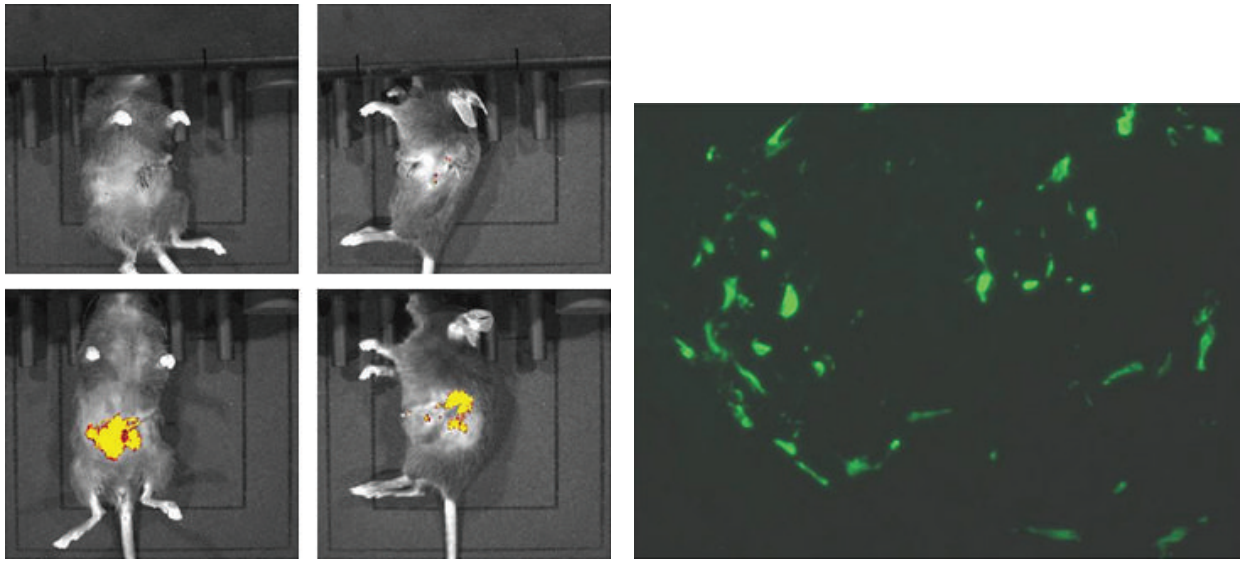

Figure 7: Migration of MSCs towards the hepatectomized liver through splenic vein. 5×10^5 Cxcr4-MSCs labeled with Vybrant DiD dye were administered to *Abcc6*$^{-/-}$; *Rag1*$^{-/-}$ mice via spleen 24 hours after partial hepatectomy, and the livers were examined at day 7 of transplantation for the homing of the transplanted cells. (a) Representative fluorescence images of hepatectomized mice after MSCs transplantation at Day 7 (lower panels). The images were examined from the ventral (left panels) and lateral (right panels) sides in comparison with PBS injected hepatectomized mice as negative control (upper panels). (b) Six-μm frozen sections were examined by immunofluoresence with an anti-GFP antibody, demonstrating the presence of GFP positive cells in the liver of mice at 7 days after intrasplenic transplantation.

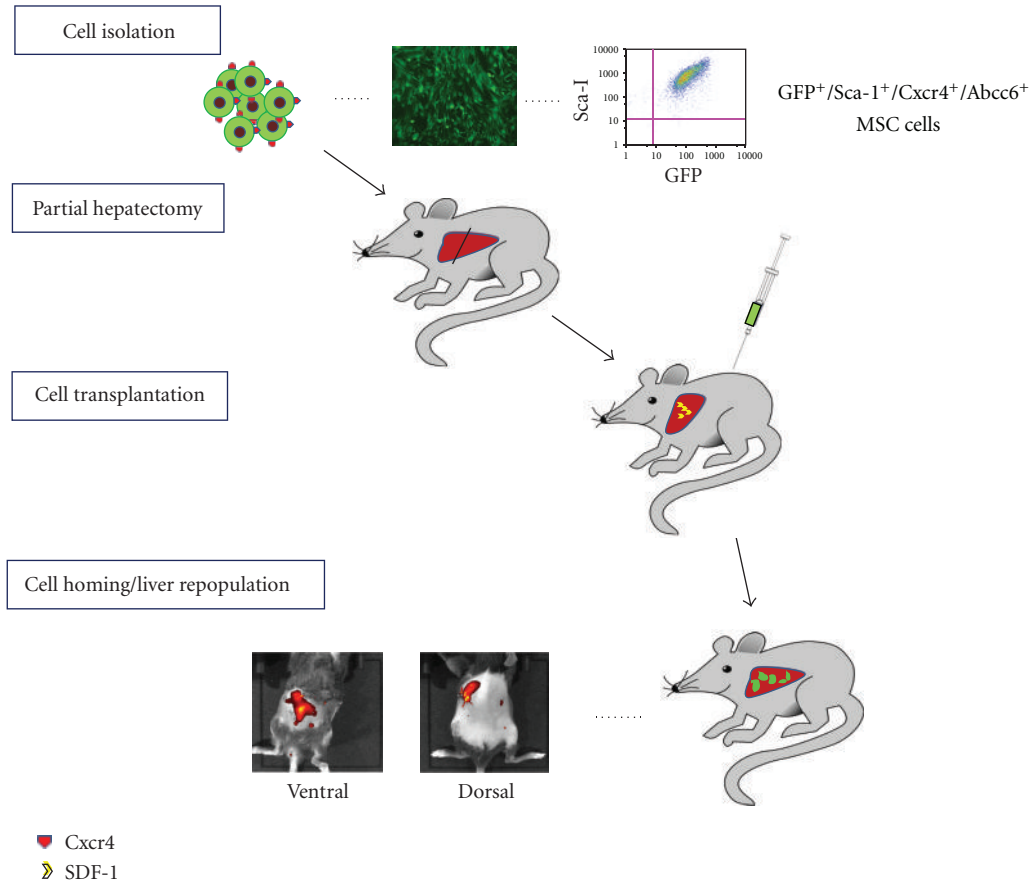

Figure 8: Schematic depiction of liver targeting of MSCs through intrasplenic injection. GFP-transgenic mouse bone marrow derived MSCs, which are positive for GFP, Sca-1, Cxcr4, and *Abcc6*, were injected through spleen 24 hours after partial hepatecotomy inducing release of SDF-1, a ligand of Cxcr4. The presence of transplanted cells was observed by a live imaging system at day 7 posttransplantation through ventral view (liver) and dorsal view (spleen).

GFP-positive Cxcr4-MSCs in the liver was 0.22%, whereas it was 0.15% in the liver from mice transplanted with MSCs without Cxcr4 transfection (data not shown).

The interaction of Cxcr4 and its ligand SDF-1 is reported to play a major role in homing of MSCs into tissues [22]. Cells in the injured organs highly express SDF-1, causing an elevation of localized SDF-1 levels, and the gradient of SDF-1 recruits MSCs into the injury site via chemotactic attraction through the interaction between SDF-1 and Cxcr4 [23, 24]. Thus, Cxcr4 has been recently used to enhance homing and engraftment of stem cells through increasing cell invasion in response to SDF-1 [23]. In this study, the local expression of SDF-1 was induced by PHx and attracted MSCs, especially Cxcr4-MSCs, toward the hepatectomized liver. Increased SDF-1 concentration was shown in the liver, but decreased SDF-1 in the blood, at 24 hours after PHx, at which time point we delivered the cells. In the mouse model of myocardial infarction, Abbott et al. reported that the SDF-1 expression increased in the peri-infarct zone, but its serum level decreased after the infarction, consistent with our result [22]. This gradient of SDF-1 may contribute to the recruitment of circulating progenitor cells expressing Cxcr4 into the hepatectomized liver (Figure 8).

4. Conclusions

The purpose of our study was to evaluate the potential of MSCs to differentiate into hepatic cells and for their efficient recruitment to the liver in the setting of partial hepatectomy. We intrasplenically delivered $Lin^{-}Sca1^{+}$ MSCs from *Abcc6*$^{+/+}$ GFP^{+} mice and identified donor cells by the presence of GFP in the liver. We did confirm the capability of MSCs to differentiate toward hepatocytic lineage *in vitro* by examining hepatic specific gene expression at mRNA and protein levels, including *Abcc6* gene expression. We also observed homing response to SDF-1, and overexpression of Cxcr4 resulted in a significant additional increase in donor-cell migration both *in vitro* and *in vivo* studies. The transplanted MSCs reside in the recipient liver for at least up to 10 days.

Over the decades, many studies have utilized bone marrow-derived cells for cell transplantation therapy in animal models of various disorders. The reported treatment effects are variable, which may be related to differences in cell type and quantity of transplanted cells, timing and approach of cell transplantation and disorder model selection. Aurich et al. demonstrated the presence of functional transplanted cells in the recipient liver at 14 weeks after administration of

human bone marrow MSCs through intrasplenic injection using an immunodeficient $Pfp^{-/-}/Rag2^{-/-}$ mouse model, which has malfunction of NK cells and depletion of mature B and T cells [25]. The long term presence of transplanted cells in recipient liver may depend on the background of recipient mice and kind of transplanted MSCs, which requires further long term study.

Immune rejection is a potential problem of cell transplantation for treatment of patients. To overcome immune rejection, or to avoid it, the stem cells must either be derived from the recipient or identical twin, or the stem cells must be engineered in a way to circumvent the immune reaction. Currently, the approach that appears most promising entails using embryonic stem cells whose DNA has been replaced with the recipient's DNA, thus becoming "self", the so-called therapeutic cloning [26].

In summary, these data suggest that purified MSCs have the capability of differentiating into hepatic lineages and homing to the liver. The interactions between SDF-1 and Cxcr4 could be a critical mechanism to recruit MSCs for liver targeting and regeneration, and further studies will define the optimal conditions to maintain and propagate MSCs in the target organs, such as in the liver in case of PXE.

Abbreviations

PXE: Pseudoxanthoma elasticum
MSCs: Mesenchymal stem cells
SDF-1: Stromal cell-derived factor 1
Cxcr4: CXC chemokine receptor 4
HSCs: Hematopoietic stem cells
PHx: Partial hepatectomy.

Conflict of Interests

The authors state no conflict of interests.

Acknowledgments

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Review Article

De Novo Kidney Regeneration with Stem Cells

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Recent studies have reported on techniques to mobilize and activate endogenous stem-cells in injured kidneys or to introduce exogenous stem cells for tissue repair. Despite many recent advantages in renal regenerative therapy, chronic kidney disease (CKD) remains a major cause of morbidity and mortality and the number of CKD patients has been increasing. When the sophisticated structure of the kidneys is totally disrupted by end stage renal disease (ESRD), traditional stem cell-based therapy is unable to completely regenerate the damaged tissue. This suggests that whole organ regeneration may be a promising therapeutic approach to alleviate patients with uncured CKD. We summarize here the potential of stem-cell-based therapy for injured tissue repair and *de novo* whole kidney regeneration. In addition, we describe the hurdles that must be overcome and possible applications of this approach in kidney regeneration.

1. Introduction

The kidney is a complex tissue consisting of several different cell types including glomerular podocytes, endothelial cells, mesangial cells, interstitial cells, tubular epithelial cells, and connecting duct cells. These cell types interact to establish a precise cellular environment that functions as an efficient tissue. The *de novo* reconstruction of the kidney is a more difficult challenge than the regeneration of many other tissues because of its complicated anatomical structure. In recent years, regenerative medicine has made remarkable progress with various groups reporting that pluripotent stem/progenitor cells have the capacity to regenerate damaged renal tissue and improve kidney function in an experimental model. However, cell-based therapy such as stem cell injection for tissue repair is not effective for the terminal stage of chronic kidney disease (CKD), which is referred to as end stage renal disease (ESRD) because of the damage that has occurred to the complex structure of the kidney including its scaffold. Currently, CKD is a serious disease worldwide that causes high mortality because of increased cardiovascular risk. The terminal ESRD stage requires renal replacement therapy and the number of ESRD patients continues to increase because of the shortage of donor organs. Consequently, more than 290,000 ESRD patients are currently undergoing dialysis in Japan.

To address this growing clinical problem, we have made a partial kidney reconstruction from mesenchymal stem cells (MSCs) in an attempt to regenerate a whole functional human kidney. In addition we have investigated the regeneration of whole kidneys in animals. Nearly all of these studies have used pluripotent stem cells, and an artificial material, blastocysts or metanephroi to act as a scaffold for the stem cells. Here, we discuss the utility of stem cells including embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, MSCs, and renal stem/progenitor cells, for the treatment of damaged renal tissue. In addition, we discuss the current advantages of *de novo* whole kidney regeneration and the obstacles that must be overcome before its clinical use is possible.

2. Embryonic Stem Cells

The first ES cells were initially derived from the inner cell mass of blastocyst-stage mouse embryos in 1983 [1]. These ES cells are pluripotent, have the ability to self-renew, and

can differentiate into several cell types of the mesodermal, endodermal, and ectodermal lineages [1]. Therefore, they have the capacity to be used as an effective tool for kidney regenerative therapy. The first human ES cell line was established by Thomson and colleagues in 1998 [2] and subsequently human ES cell lines have been found to be capable of differentiating *in vitro* into extraembryonic and somatic cell lineages [3]. If human ES cells are cultured with a mixture of eight growth factors (basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), activin-A, bone morphogenetic protein-4 (BMP-4), hepatocyte growth factor (HGF), epidermal growth factor (EGF), β-nerve growth factor (β-NGF), and retinoic acid) they will differentiate into cells expressing WT-1 and renin [4]. In addition, it has been shown that mouse ES cells stably transfected with Wnt4 will differentiate into tubular-like structures that express aquaporin-2 when cultured in the presence of HGF and activin-A [5]. The combination of LY294002, CCG1423, and Janus-associated tyrosine kinase inhibitor 1, was shown to enhance the differentiation of mouse ES cells into a pool of renal progenitor cells and intermediate mesoderm [6]. Steenhard et al. investigated an *ex vivo* culture system, in which ES cells were microinjected into the developing metanephros and this was cultured to determine the capacity of ES cells to differentiate into renal cells. They identified renal epithelial structures that resembled tubules with an efficiency approaching 50% and on rare occasions, individual ES cells were observed in structures resembling glomerular tufts [7]. In addition, when ES cells, treated with retinoic acid, activin A, and BMP-7, were injected into a developing metanephros, they contributed to the tubular epithelia with almost 100% efficiency [8]. The injection of ES cells with brachyury (T) expression into developing metanephros explants in organ culture, resulted in their incorporation into the blastemal cells of the nephrogenic zone. After a single injection into a developing, live, newborn mouse kidney, these cells were integrated into the proximal tubules with normal morphology and polarization of alkaline phosphatase and aquaporin-1 [9]. On the other hand, we recently reported that the *in vitro* culture of monkey ES and human iPS cells in rat metanephros showed teratoma formation [10]. In considering the therapeutic approaches using human ES cells two major issues arise. One issue is the ethical concerns surrounding the use of donated eggs to establish ES cells, and the other is the immune rejection due to histocompatibility antigenic differences between the ES cells and patients [11]. In summary, ES cells are a valuable cellular source for investigating the mechanism of cell development, but are unsuitable for clinical applied regeneration therapy.

3. Induced Pluripotent Stem Cells

Takahashi and Yamanaka have reported the generation of induced pluripotent stem (iPS) cells from murine somatic cells by retroviral transfer of expression constructs for the transcription factors Oct3/4, Sox2, Myc, and Klf4 [12]. Similarly, iPS cells have been established from several mammalian species, including rat [13, 14], rabbit [15], pig [16, 17], monkey [18], and human [19]. The generation of iPS cells has recently been reported from human mesangial cells [20], urine [21], and tubular cells [22]. In fact, iPS cells can be established without transfection of Klf4 [22] and Myc, which is a oncogenic factor [22, 23]. These data suggest that the oncogenic risk associated with iPS cell generation can be decreased by expressing only Oct3/4 and Sox2. Therefore, it is possible to prepare patient-specific pluripotent cells without manipulating germ cells because iPS cells are pluripotent and can be generated from adult somatic cells. Consequently, there are no ethical issues with the usage of iPS cells and immune rejection should not be a problem compared to ES cells. Potentially, iPS cells could provide a source of cells for kidney tissue repair or organ regeneration, although the difference between ES cells and iPS cells in their regenerative capacity to become kidney tissue has not yet been elucidated. The therapeutic potential of autologous iPS cells in a mouse model of hereditary disease has already been reported [24]. Therefore, the generation of iPS cells may open the door for a new autologous stem cell therapy for kidney regeneration. One recent study has indicated that the transplantation of iPS cells, but not ES cells, induces a T-cell-dependent immune response even in a syngeneic mouse [25]. These data contradict the concept of using iPS cells for regenerative medicine and therefore we need to evaluate the indications for iPS cells before they can be used in a clinical application.

4. Mesenchymal Stem Cells (MSCs)

Since 2000, bone marrow-derived stem cells (BMDCs) have been used in experimental kidney disease models because of their ability to differentiate into organ-specific cell types and regenerate several parts of the kidney. Several studies have indicated that treatment with BMDCs can ameliorate several injured renal tissues: tubular epithelial cells [26, 27], mesangial cells [28–30], podocytes [31, 32], and endothelial cells [33–35]. BMDC treatment can contribute to the attenuation of renal fibrosis during chronic renal disease progression [36]. However, donor BMDC migration into the kidney is very rare and their ability to transdifferentiate is limited. It is possible that the benefit of administering BMDCs is only derived from the paracrine action of the injected cells [37, 38]. Bone marrow includes hematopoietic stem cells (HSCs), MSCs, and endothelial progenitor cells and the use of selected populations of BMDCs such as MSCs has been proposed [39, 40]. The injection of bone marrow-derived MSCs can result in repair of the kidney and improve function in acute renal failure. Furthermore, several studies have shown that MSCs derived from kidney [41] and adipose tissue [42] instead of bone marrow represent a source of cells for the improvement of damaged renal tissue and function. These studies suggest that the presence of BMDC-derived kidney component cells is most likely due to the MSC population, which are adult stem cells with the capacity for self-renewal and multipotent differentiation. MSCs also produce cytokines such as vascular endothelial growth factor (VEGF), HGF, and insulin-like growth factor-1 (IGF-1) [43] that inhibit the profibrotic activity of TGF-β, which is a major factor for that epithelial-mesenchymal

transition (EMT) that leads to kidney fibrosis [44] and causes CKD. These humoral factors act to reduce inflammation and repair damaged kidney tissue. VEGF resolves glomerular inflammation, enhances glomerular capillary repair [45], induces endothelial cell proliferation, and prevents the loss of peritubular capillaries [46]. HGF inhibits epithelial cell death and accelerates regeneration and remodeling of damaged renal tissue [47]. IGF-1 secreted by MSCs accelerates tubular cell proliferation and aids the function and repair of injured renal tissue [48]. The conditioned media obtained from cultures of MSCs induces the migration and proliferation of kidney-derived epithelial cells and diminishes proximal tubule cell death [49]. These investigations show that the improvement of kidney function associated with MSC treatment is most likely caused by the secretion by MSCs of humoral factors that act on the injured tissue.

More recently, MSCs have been used in acute renal injury models but also the treatment of CKD [50, 51], diabetic nephropathy (DN) [52–55], and in a chronic allograft nephropathy model [56]. MSC treatment has also reduced renal fibrosis and ameliorated renal function in a rat remnant kidney model [50]. The levels of all cytokines in serum were decreased in MSCs-treated CKD rats, which suggests that MSCs therapy can indeed modulate the inflammatory response and suppress kidney remodeling in chronic kidney disease. In the same way, injected MSCs regulated the immune response that resulted in the acceleration of glomerular tissue repair and an improvement in kidney function in DN model rats [52–55]. It has also been observed that the injection of MSCs 11 weeks after kidney transplantation prevents interstitial fibrosis [56]. These data suggest that MSCs transplantation can inhibit the progression of DN and CKD and improve allograft renal function in both animals and humans. In contrast, MSCs can maldifferentiate into glomerular adipocytes accompanied by glomerular sclerosis [57] thus calling into question the benefit of long-term MSC treatment for chronic glomerular disorders [58]. Furthermore, recent reports suggest that the administration of external stem cells has additional risks in a clinical setting [59, 60].

Noh et al. reported that uremia induces functional incompetence of bone marrow-derived MSCs in an animal model [61]. Uremic MSCs showed decreased expression of VEGF, VEGF receptor 1, and stromal cell-derived factor (SDF)-1α, increased cellular senescence, decreased proliferation, defects in migration in response to VEGF, and SDF-1α and tube formation *in vitro* [61]. This study suggests that MSCs from CKD patients may be inappropriate as a source of cells for regeneration therapy. Further research is required to evaluate and solve the problems associated with regeneration therapy in order to make safe and effective use of MSC for kidney regeneration.

5. Renal Stem/Progenitor Cells

Adult stem/progenitor cells have been isolated from many adult organs that have clonogenic, self-renewing ability and will give rise to terminally differentiated cells of original tissue. Renal stem/progenitor cells exist in the adult kidney and are located in specific locations such as the renal papilla [62], tubular epithelial cells [63], Bowman's capsule [64], and the S3 segment of the proximal tubules [65, 66].

A number of different approaches have been made in the investigation of the functional role of renal stem/progenitor cells in the adult kidney [67]. The evidence for the presence of renal stem cells in adult kidney has relied upon the presence of cells positive for bromodeoxyuridine (BrdU), specific cell surface markers such as CD133 and CD24, or side population (SP) phenotypes. The first approach utilized a short pulse administration of BrdU followed by a long chase period. The rationale for this was based on the characteristically slow cycling time of organ-specific adult stem cells [68–70]. Stem cells incorporate BrdU into their DNA and retain this label to enable detection for an extended period of time [71, 72]. In normal rat kidney, cells that retain the BrdU label can be detected in papilla [62], as well as proximal, distal, and collecting tubules [63]. These cells proliferate in response to renal damage and differentiate into fibroblasts [73], proximal tubule and collecting duct cells as well as tubular structures *in vitro* [74]. Although more likely to represent stem cells, clonogenicity of these cells was not established [75].

An additional approach to studying renal stem/ progenitor cells is based on the analysis of stem-cell specific surface markers. Recent studies have been reported that a population of $CD133^+/CD24^+$ cells, in the absence of the podocyte marker, podocalyxin (PDX) are located at the urinary pole of the Bowman's capsule. This is the only place in the human kidney that appears to be contiguous with both tubular cells and glomerular podocytes [58, 64, 76–78]. Clonally-expanded $CD133^+/CD24^+/PDX^-$ progenitor cells are multipotent and are capable of differentiating into podocytes and tubular cells *in vitro* [76]. This population also contributes to the regeneration of podocytes and tubular cells after injection into mice with acute renal failure [58, 76].

Analysis of side population (SP) phenotypes has been adopted as another approach to identify renal stem cells in fractionated whole kidney. The term SP is used to describe HSCs that are isolated by using dyes such as Hoechst 33342 and Rhodamine 123 because HSCs have the ability to efflux these dyes. Cells with the same efflux profile in kidney may also a similar organ-based SP phenotype and function as organ-specific stem cells [75]. SP cells have been reported to present in the adult rodent kidney [79–82], and adult kidney SP cells show multilineage differentiation *in vitro.* The injection of adult kidney SP cells reduces renal damage without significant tubular integration [82, 83]. These data reveal that humoral factors may be important for amelioration of renal injury. However, it remains unclear whether kidney-derived SP cells are in fact renal stem cells, because their capacity for self-renewal has not been established [75].

Lindgren et al. recently demonstrated that aldehyde dehydrogenase (ALDH) activity can be used as a marker for isolation of cells with progenitor characteristics from adult human renal tissue [66]. Primary renal cortex cells with high ALDH activity were isolated by fluorescence-activated cell sorting (FACS) and express CD24 and CD133, which

are previously described markers of renal progenitor cells of Bowman's capsule. Functional and bioinformatic analyses of these cells showed that they have a robust phenotype that allows an increased resistance to acute kidney injury and suggests that these cells may spearhead the repopulation of renal tubules after injury.

A number of questions regarding the use of renal stem/progenitor cells in regenerative therapy remain to be answered. These include whether endogenous renal stem cells can be identified efficiently, whether they can be expanded *in vitro* and redelivered to a damaged kidney. Renal stem cell represents only 0.1% of the cells in an adult kidney [75, 82]. Therefore, whole kidney fractionation was necessary to produce sufficient renal stem/progenitor cells in these recent reports. Renal stem/progenitor cells differentiated from extrarenal stem cells such as MSCs, ES, and iPS cells may be promising cellular sources for kidney repair. However, a reliable method of inducing extrarenal stem cells to differentiate into renal progenitor cells has not been established at this time.

6. Other Stem Cells

Recent studies have reported that multilineage-differentiating stress-enduring (Muse) cells were isolated from human dermal fibroblasts. Muse cells are characterized by stress tolerance, expression of pluripotency markers, self-renewal. In addition, they have the ability to differentiate from a single cell both *in vitro* and *in vivo* into endodermal, mesodermal, and ectodermal cells [84]. Muse cells may also have the possibility to regenerate injured renal structure and further study of their use in regeneration therapy is required.

7. *De Novo* Organ Regeneration

7.1. Organ Regeneration Using Bioengineered Scaffolding. Advances in biomaterial engineering have produced bioengineered scaffolds that facilitate improved differentiation of transplanted cells. Tissue-engineering strategies combining artificial scaffolds and stem cells have been adapted for kidney regeneration. Lanza et al. initially reported that a histocompatible functional kidney was generated by using a specialized polymer tube as the artificial scaffold [85]. They used a nuclear transplantation technique in which dermal fibroblasts isolated from an adult cow were transferred into enucleated bovine oocytes and then transferred nonsurgically into progestin-synchronized recipients. Metanephroi from embryos were digested using collagenase, and the cells were expanded *in vitro* until the desired number was produced. The cells were then seeded onto a specialized polymer tube, which was implanted into the same cow from which the cells had been cloned. This renal device that was seeded with cloned metanephric cells appeared to produce a urine-like liquid. Histologic analysis showed that the device had well-differentiated kidney-like construction. This included organized glomerulus-like, tubular-like, and vascular elements, which were clearly distinct from each other, but were continuous within the structure. The kidney-like structure appeared to be integrally connected in a unidirectional manner to the reservoirs, resulting in the excretion of urine into the collection system. This study established that bioengineered tissue scaffolds are potential tools for kidney regeneration.

7.2. Organ Regeneration Using Decellularized Cadaveric Scaffolds. Recent studies have reported that a decellularized organ can be useful as an artificial scaffold. The decellularization process preserves the structural and functional characteristics of the native microvascular network. Ott et al. showed the successful development of a functional artificial rat heart using a decellularized cadaveric heart as the artificial scaffold [86]. A whole-heart scaffold with intact three-dimensional geometry and vasculature was prepared by coronary perfusion with detergents into the cadaveric heart. This heart was then colonized by neonatal cardiac cells or rat aortic endothelial cells and cultured under physiological conditions to promote organ development [86]. The injected neonatal cardiac cells produced a contractile myocardium, which performed the stroke function.

Cadaveric scaffolds have also been investigated to develop transplantable livers and lungs using mature hepatocytes and alveolar epithelial cells, respectively [87, 88]. After transplantation of the recellularized grafts, they successfully functioned as hepatocytes and gas exchangers, respectively. This type of approach is promising for regenerating organs that have a simple architecture.

Based on a series of studies, Ross et al. successfully regenerated an entire kidney using a decellularized cadaveric kidney scaffold [89]. After decellularization of an intact rat kidney, murine ES cells were injected into the renal artery where they localized in the vasculature, glomeruli, and tubules. Immunohistochemical analysis indicated that the injected ES cells had lost their embryonic appearance and had developed to mature kidney cells. This approach was supported using the primate kidney [90] but the regenerated primate kidney did not have sufficient renal function to produce urine and erythropoietin (Epo). Therefore the reconstruction of a whole functional kidney may be difficult using this approach.

8. *De Novo* Organ Regeneration Using Blastocyst Complementation

Recently, a dramatic advance has been made in pancreas regeneration using the interspecific blastocyst injection of iPS cells [91]. When rat iPS cells were injected into $Pdx1^{-/-}$ (pancreatogenesis-disabled) mouse blastocysts, the newborn rat/mouse chimera possessed a pancreas derived almost entirely from rat iPS cells. This result shows that when an empty developmental niche for an organ is provided, then iPS cell-derived cellular progeny can repopulate that niche and can develop into the missing contents of the niche. In fact, they can form a complicated organ that is composed almost entirely of cells differentiated from donor iPS cells, even if the blastocyst complementation is derived from a different species.

Espejel et al. generated chimeric mice in which all of the hepatocytes were derived from iPS cells from blastocysts with

fumarylacetoacetate hydrolase deficiency [92]. The entire liver was composed of iPS cell-derived hepatocytes by the time the mice reached adulthood. iPS cells have the intrinsic ability to differentiate into fully mature hepatocytes that provide full liver function. The iPS cell-derived hepatocytes also replicated the unique proliferative capabilities of normal hepatocytes.

This blastocyst complementation system was recently applied to whole kidney reconstruction [93]. Murine iPS cells were injected into blastocysts from mice that did not express the SAL-like 1 (Sall1) zinc-finger nuclear factor essential for kidney development. The newborn mice possessed kidneys derived almost entirely of injected iPS cells. While this is an attractive system, it is not available for clinical use because it is impossible to generate the vascular and nerve systems. In addition, immunohistochemical analysis of the regenerated kidney indicated that the renal vascular system including renal segmental, lobar, interlobar, arcuate, and interlobular arterioles was a chimeric structure originated from both host cells and donor iPS cells [94]. When rat iPS cells were injected into Sall1-null mice blastocysts, they did not generate rat kidneys in mice. This suggests that the key molecules in mice involved in the interactions of the mesenchyme and the ureteric buds do not cross-react with those in rats. Therefore, to generate xenoorgan using xenoblastocysts, it would be necessary to generate a host animal strain lacking all of the lineages that contribute to the kidney [93]. At present the most important ethical issues involved with manipulating heterogeneous blastocysts containing iPS cells remain unresolved. In addition, while it is quite difficult to generate interspecific chimeras in animals, blastocyst complementation appears to be one of the most promising strategies for regenerating the kidney.

9. *De Novo* Organ Regeneration Using the Metanephros of Growing Xenoembryos

The embryonic metanephros is a primordium of the adult mammalian kidney and represents a source for a transplantable artificial kidney [95–99]. Metanephroi implanted into a host renal cortex or omentum continue to develop and enlarge. The differentiated metanephroi in a host animal have vascularized glomeruli and mature proximal tubules and produce urine [95, 96]. After an intact ureteroureterostomy, anephric rats with a transplanted metanephros show prolonged lifespan [96]. The transplanted metanephros is also metabolically functional and produces Epo and renin, as well as elevates the blood pressure of the host animal [100, 101]. Furthermore, porcine metanephroi transplanted into the omentum of mice treated with costimulatory blockade [97] or transplanted under the kidney capsules of immunodeficient mice [98], also differentiated into a functional nephron. The levels of urea nitrogen and creatinine were higher in the cyst fluid produced by the transplanted tissue, than in the sera of the transplanted mice [98]. This suggests that the metanephros is a potential source of transplantable regenerated kidney to address the shortage of organs for kidney transplantation.

We have attempted to regenerate a whole functional kidney using a developing heterozoic embryo as an organ factory. We sought to use this mechanism of a developing embryo by applying the stem cells at the niche of organogenesis. During development of the metanephros, the metanephric mesenchyme (MM) initially forms from the caudal portion of the nephrogenic cord [102] and secretes glial cell line-derived neurotrophic factor (GDNF), which induces the nearby Wolffian duct to produce a ureteric bud [103]. Therefore, we microinjected GDNF-expressing human MSCs (hMSCs) into the site of budding. The recipient embryo was grown in a whole embryo culture system, and the metanephros that formed was developed in organ culture [104, 105]. Virus-free manipulation can also be performed using thermoreversible GDNF polymer [106]. Donor hMSCs were found to be integrated into the rudimentary metanephros and morphologically differentiated to tubular epithelial cells, interstitial cells, and glomerular epithelial cells [104]. These data indicate that using a xenobiotic developmental process for growing embryos allows endogenous hMSCs to undergo an epithelial conversion and develop into an orchestrated nephron including glomerular epithelial cells and tubular epithelial cells. The hMSCs can also differentiate into renal stroma after renal development [104].

We then examined whether there was urine production from the "neokidney," which is of major importance for successful *de novo* renal regeneration. Urine production requires that the new kidney has the appropriate vascular system of the recipient. Therefore, we transplanted metanephroi into the omentum in order to allow for vascular integration from the recipient to form a functional nephron. As a result, an hMSC-derived neokidney was generated that contained a human nephron and the vasculature from the host [105, 107]. In addition, the neokidney produced urine that showed higher concentrations of urea nitrogen and creatinine than the sera of the recipient. This suggested that the neokidney that developed in the omentum was capable of producing urine by filtering the recipient's blood [107]. Furthermore, the hMSC-derived neokidney secreted human Epo, which was stimulated by the induction of anemia in the host animal, indicating that this system preserves the normal physiological regulation of Epo levels [108].

The current system we have developed may not reconstruct derivatives of the ureteric bud. Thus we sought to determine whether MSCs can differentiate into the ureteric bud progenitor using chick embryos. The hMSCs that expressed Pax2 were injected into the chicken ureteric bud progenitor region and they migrated caudally with the elongating Wolffian duct [109]. The hMSCs were integrated into the Wolffian duct epithelia and then expressed LIM1, revealing that they can differentiate into the Wolffian duct cells under the influence of local xenosignals [109]. These results indicate it might be possible to rebuild the whole kidney by transplanting hMSCs at a suitable time and place to regenerate derivatives of the MM and ureteric bud.

We recently reported that the xenotransplanted metanephros provides a niche for endogenous MSC differentiation into Epo-producing tissue [110]. Xenotransplanted metanephros, from rat into mouse and similarly from pig

into cat, expresses Epo of the host animal origin, as shown by PCR using species specific primers and sequence analysis. This suggests that there has been recruitment of host cells and Epo production. The Epo-producing cells were not differentiated from integrating vessels because they did not coexpress endothelial markers. Instead, Epo-producing cells were revealed to be derived from circulating host cells, as shown by enhanced green fluorescent protein (EGFP) expression in the grown transplants of chimeric mice bearing bone marrow from a transgenic mouse expressing EGFP under the control of the Epo promoter. These results suggest that donor cell migration and differentiation in a xeno-transplanted developing metanephros may be consistent between species. The Epo-producing cells were identified as MSCs by injecting human bone marrow-derived MSCs and endothelial progenitor cells into NOD/SCID mice. Furthermore, using metanephroi from transgenic ER-E2F1 suicide-inducible mice, the xenotissue component could be eliminated, leaving autologous Epo-producing tissue. Our findings may alleviate adverse effects due to long-lasting immunosuppression and help mitigate ethical concerns. These data suggest that xenometanephroi can provide the niche for host bone marrow cells to differentiate into Epo-producing tissues and they can be reconstructed to consist exclusively of host cell components using fate-controlled animals.

10. *In Vitro* Kidney Regeneration without any Scaffolding

A number of research groups are investigating whether pluripotent stem cells can differentiate into a kidney structure without any external scaffold. ES or iPS cells have been differentiated into mature cell types in adult organs, such as the pancreas [111, 112], liver [113, 114], and intestine [115] by using stepwise protocols mimicking the mechanism of embryonic development. In order to regenerate insulin-producing cells, ES [111] or iPS cells [112] were first differentiated into definitive endoderm, then foregut endoderm, followed by pancreatic progenitors, and eventually insulin-expressing endocrine cells. On the other hand, recent studies have revealed that autonomous formation of three-dimensional adenohypophysis [116] and optic cap [117] structures in aggregate culture of pluripotent ES cells. Osafune et al. previously established that a single cell from the MM, which highly expresses Sall1, can form colonies and reconstruct a three-dimensional kidney structure composed of glomeruli and renal tubules [118]. A recent study also established a novel method in which embryonic kidneys are dissociated into single-cell suspensions and then reaggregated to form organotypic renal constructions [119]. These investigations suggest the possibility of establishing a whole kidney from pluripotent stem cells by using the stepwise differentiation approach. This would involve initially directing the pluripotent stem cells to form intermediate mesoderm, then renal progenitors [11]. As a result the three-dimensional kidney structure could also be developed from these pluripotent stem cells *in vitro*.

The signals involved in embryonic kidney development have not yet been fully revealed and the technique required for the induction of iPS cell differentiation into renal cells remains uncertain at this time. Furthermore, the route for the reconstruction of the renal vascular system between the regenerated kidney and the recipient remains unclear. Therefore, this area requires additional research and further advances in stem cell biology will enable the development of new therapeutic strategies for the treatment of renal diseases.

11. Conclusions

We have summarized recent advances in renal regenerative therapy including the potential of stem cells to treat damaged renal tissue and to regenerate a whole organ *de novo*. At this time, the utilization of stem/progenitor cells for regeneration therapy has both advantages and disadvantages. Even though ES cells are pluripotent, there are ethical problems associated with the manipulation of germ cells in producing ES cells. Similarly, iPS cells are pluripotent but the use of retroviral transduction and our limited understanding of its effects hinder the clinical potential of iPS cells. The use of renal stem/progenitor cells in kidney regeneration is limited by their restricted growth and differentiation potential as well as their low prevalence. Therefore, renal stem/progenitor cells appear to be unsuitable for whole kidney regeneration. In contrast, MSCs are easily accessible, especially from adipose and do not require technical manipulations. However, MSCs from CKD patients may be inappropriate for regeneration therapy, because uremia induces functional incompetence of MSCs. On the other hand, recently, new findings against this opinion have been reported [120]. The determination of the optimal source of cells for *de novo* kidney regeneration remains an important aim.

On the other hand, we make effort to regenerate *de novo* a whole functional kidney by using xenoembryos and have investigated successful reconstruction of a part of a functional kidney derived from hMSCs, because of the necessity of *de novo* development of an entire functional organ for ESRD patients. Based on this success, we are currently investigating whether the pig is suitable for our system, because the porcine kidney is almost the same volume as the human kidney [98]. Even though kidney regeneration using heterologous animals, such as xeno-decellularized cadaveric organ, xenoblastcyst, and xeno-embryos, is a promising strategy, the ethical issues remain controversial. However, we hope that this system in larger animals will facilitate the development of larger organs that are more suitable for use in humans and make effort to solve the shortage of organ donors.

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Review Article

Development of New Technologies for Stem Cell Research

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Since the 1960s, the stem cells have been extensively studied including embryonic stem cells, neural stem cells, bone marrow hematopoietic stem cells, and mesenchymal stem cells. In the recent years, several stem cells have been initially used in the treatment of diseases, such as in bone marrow transplant. At the same time, isolation and culture experimental technologies for stem cell research have been widely developed in recent years. In addition, molecular imaging technologies including optical molecular imaging, positron emission tomography, single-photon emission computed tomography, and computed tomography have been developed rapidly in recent the 10 years and have also been used in the research on disease mechanism and evaluation of treatment of disease related with stem cells. This paper will focus on recent typical isolation, culture, and observation techniques of stem cells followed by a concise introduction. Finally, the current challenges and the future applications of the new technologies in stem cells are given according to the understanding of the authors, and the paper is then concluded.

1. Introduction

Stem cells are a kind of cells that have the ability to perpetuate themselves through self-renewal and to generate functional mature cells of a particular tissue through differentiation [1, 2]. So far, stem cells do not have a clear definition which can be used to distinguish them from other cells. However, most of researchers believe that, in general sense, stem cells need to meet the following criteria. First of all, stem cells are capable of continuous, repeated self-renewal divisions in order to maintain the stem cell population. Secondly, a single stem cell can differentiate into a variety of mature cells. Thirdly, stem cells can rebuild the organization when they are transplanted into its source damaged organization. Lastly, even if the tissue does not suffer, stem cells can differentiate into mature cells of the tissue.

In recent years, tumor stem cells increasingly attracted much more attention of researchers. Many researchers found that malignant tumor growth, recurrence, and metastasis were similar to the corresponding characteristics of stem cells [1–3]. With the deepening of research on stem cell and tumor biology, tumor stem cells are initially found in acute myeloid leukemia (AML) [4–8]. Park and his partners had proved that only a small part of the cell subsets of leukemia and multiple myeloma cells have unlimited proliferative capacity, accounting for 1%–4% and 0.001%–1% of the total number of tumor cells, respectively [4]. Salmon's group had successfully identified and separated the tumor stem cells with CD34 (+)/CD 38 (−) phenotype from the human acute myeloid leukemia tumor cells for the first time [5]. Afterwards, Morrison's and Licht's groups had done some researches on the targets of the AML and this work would surely promote the development of clinical treatment of AML [6–8].

For stem cell research, some technologies are frequently used including stem cell culture, separation, and identification techniques. At present, fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) are the common separation methods for stem cells. Among them, FACS is the most widely used method. Researchers had obtained the cancer stem cells from breast cancer, brain gliomas, prostate cancer [9], gastric cancer [10], lung cancer [11], liver cancer [12], and pancreatic cancer [13, 14] using this method. In addition, some cell labeling technique had also been applied in stem cell research. Perrimon's group had labeled the cell using site-specific recombinant technique

to randomly activate the lacZ gene in cell population [15]. Through labeling some specific gene, function of the stem cell could be detected and identified [16]. Lastly, with the advances in microscopy, confocal microscopy techniques have been developed and used to observe the stem cells and their surroundings.

In 1999, Weissleder and his partners proposed the concept of molecular imaging at Harvard University [17]. Through 10 years of development, this technology has been widely applied to many aspects of the life sciences. The goal of molecular imaging is to depict noninvasively *in vivo* cellular and molecular processes sensitively and specifically, such as monitoring multiple molecular events, cell trafficking and targeting [18–20]. In recent years, some new algorithms especially in optical molecular imaging area have been developed, such as multilevel adaptive finite element method [18], Bayesian approach [21], and graph cuts [22]. At the same time, with the advances of hardware and machine technique, some new molecular imaging systems had been developed, such as Spectrum CT of Cold Spring Biotech Corporation with three-dimensional imaging functional. In addition, Cerenkov luminescence imaging (CLI) using Cerenkov theory had been developed to capture the signals derived from ^{18}F-FDG probe. Compared to the traditional imaging method, all these imaging technologies had shown its advantage in tumor mechanism and antitumor evaluation research and had been initially applied in the stem cell research.

In this review paper, we will focus on the following three aspects. Firstly, we will introduce some new isolation and culture technology and review its application in stem cell research. Secondly, we will introduce several molecular imaging techniques and review some researchers' work on stem cells using these techniques. Finally, we will predict the future prospects to conclude the paper.

2. New Isolation and Culture Technology and Its Application in Stem Cells Research

Traditional culture methods typically involve reprogramming somatic cells to pluripotency by serial passage under adherent culture conditions on feeder cells or on extracellular matrix compounds [25]. The stem cells using these approaches likely to be contaminated by pathogens; these approaches are require separation of feeder cells from the cell type of interest which increase costs and are prone to differentiate variability. In recent years, many new isolation and culture technologies have been developed to obtain stem cells for their wide application prospects in disease mechanism and its treatment including suspension technology [23] and SB431542 inhibitor differentiation method [24].

2.1. Suspension Technology. Induced pluripotent stem cells (iPSCs) draw more and more attention because of their therapeutic advantages in enabling the generation of high-quality disease models, derivation of individual-specific iPSC lines, improving the predictability of drug action, and as a source of cells for regenerative medicine [23]. Zandstra's group had developed a way to obtain induced pluripotent stem cells in continuous adherence- and matrix-free suspension culture system, which has the potential to accelerate and standardize iPSCs research. The gene expression analysis showed high correlation between the two processes including reprogramming in suspension culture and that in routine adherent culture with regard to hallmark reprogramming genes.

2.2. SB431542 Inhibitor Differentiation Method. Mesenchymal stem cells are one kind of adult stem cells, which mainly derived from bone marrow stromal cells. Because of their ability to differentiate into a wide range of mesenchymal-lineage tissues, mesenchymal stem/stromal cells (MSCs) are under intense investigation for applications in cardiac, renal, neural, joint, and bone repair, as well as in inflammatory conditions and hemopoietic cotransplantation [24]. MSCs are typically harvested from adult bone marrow or fat, but these methods not only require painful invasive procedures, but also are low-frequency sources, with MSCs making up only 0.001% of bone marrow cells and 0.05% in fat tissues [26, 27].

Traditional fetal MSCs isolation method was described in Guillot's paper [28]. Briefly, first-trimester fetal bone marrow MSCs were obtained by flushing the bone marrow cells out of humeri and femurs using a syringe and a 22-gauge needle. The resultant cells were allowed to adhere to a standard tissue culture flask for 72 hours in MSC medium. Afterwards, they were washed and passaged with Tryple-Select upon confluence, cultured under humidified conditions in 5% CO_2, and routinely cryopreserved in 90% FCS and 10% dimethyl sulfoxide (DMSO). Fisk's group had developed a new method to obtain MSCs and this research result would influence deeply a number of patients suffering from serious disease. In their studies, firstly, to develop a feeder cell-free method to produce MSCs from ESCs or iPSCs, they used a widely used commercially available defined medium, mTeSR1(Stem Cell Technologies), which in combination with the cell attachment matrix Matrigel, maintains pluripotency of ESCs/iPSCs without the need for feeder cells or additional basic fibroblast growth factor (bFGF) [29]. Then cells were seeded as large colonies at high confluence, and some cell lines required a one-passage adaptation to mTeSR/Matrigel conditions prior to MSC differentiation. When cells were confluent, the medium was changed to inhibitor differentiation medium including knockout serum replacement (KOSR) medium without bFGF, supplemented with 10 μM SB431542(SB) (a kind of transforming growth factor-β) in DMSO. The MSCs could be obtained using this new method in about 10 days, which was more quickly than traditional methods.

In addition to the two new methods developed recently, many researchers had done much work on the isolation and culture technology of stem cells in order to apply it in the treatment of disease more extensively. Fan's group had observed [30] that the embryonic stem cells possessed a relatively loose, open chromatin structure, and the differentiated cells possessed a tight chromatin structure. This research demonstrated for the first time that the state of chromatin compaction was not only the result of cell differentiation,

but also the cell differentiation needed. Fischbach's group had showed that adipose-derived stem cell could trigger the tumor growth [31]. Yamanaka's group had induced pluripotent stem cells from mouse embryonic [32] and adult human fibroblasts using defined factors [33]. All these studies had promoted the stem cells' application in the treatment of disease including tumor and regenerative medicine.

3. Molecular Imaging Techniques and Their Application in Stem Cell Research

Molecular imaging, including bioluminescence imaging (BLI), fluorescence molecular imaging (FMI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI) can characterize and quantify biological process at the cellular and molecular level in intact living subjects. It usually exploits specific molecular probes as the source of image contrast to detect the disease and evaluate drug efficacy [34]. In recent years, with the development of molecular imaging including imaging systems and imaging algorithms, molecular imaging has been widely applied in many areas, such as tumor research, drug development, and stem cell research and so on. Take the bioluminescence imaging system as an example, it has been developed from two-dimensional system to three-dimensional systems. Fluorescence imaging system has also been greatly improved in imaging acquisition speed and stability. Meanwhile, many algorithms had been developed to improve the speed and accuracy of image reconstruction, such as Tikhonov regularization method [18], a Born-type approximation BLT method [35], the Bayesian approach [21]. The development in system and algorithm enables molecular imaging to become an important means of stem cell research. Then we will review some researches of molecular imaging on tracking stem cell therapy of cardiovascular and neurological diseases.

3.1. Molecular Imaging for Tracking Stem Cell Therapy in Cardiovascular. Cardiovascular disease is the second cause of morbidity and mortality in China, and the leading cause of morbidity and mortality in the United States [38]. One major reason for the high morbidity and mortality is that the heart has an inadequate regenerative response following ischemia caused by myocardial infarction or other chronic cardiovascular diseases [39, 40]. Novel regenerative therapies like stem cell therapy can promote neovascularization and neomyogenesis, which need to be evaluated using molecular imaging [41, 42].

For cardiac stem cell therapy, Bulte's group monitored the trafficking of ^{111}In labeled mesenchymal stem cells after intravenous administration in a porcine myocardial infarction model using SPECT imaging [43]. Cao's group demonstrated the utility of BLI by tracking survival and proliferation of mouse ESCs following cardiac injection in rats in 2006 [44]. Then Li's group made a head-to-head comparison of BLI and MRI using human ESCs in immunodeficient mouse hind limb models and found that MR images showed stable and similar signals in both undifferentiated ESCs and differentiated endothelial cells for 4 weeks, whereas BLI showed divergent survival profiles for the two groups [36]. The result was shown in Figure 1. In addition, Schrepfer's group has used GFP to histologicaly verify the presence of transduced bone marrow mononuclear cells following transplantation into myocardium [45]. All these imaging technologies including BLI, FMI, PET, SPECT, and MRI have been used in tracking stem cell therapy in cardiovascular, which will promote the development of stem cell therapy.

3.2. Molecular Imaging for Stem Cell Therapy in Neurological Diseases. The nervous system is a delicate and complex system, composed of neurons, glial cells, microglia, and cells and blood vessels of the meninges. Human neurological diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, spinal cord injury, and multiple sclerosis are caused by loss of different types of neurons and glial cells in the brain and spinal cord [46]. Discovery of the therapeutic potential of stem cells offers new methods for the treatment of neurological diseases. Advances in imaging equipment and technique offer powerful methods for evaluating therapeutic efficacy of neurological diseases [47, 48].

For PET imaging, as a common imaging probe, ^{18}F-FDG has been used to label porcine circulation progenitor cells with the labeling efficiency 91.6 ± 6.4% [49]. Furthermore, Kang's group has evaluated the efficacy of stem cell therapy in human heart using PET in clinical studies [50, 51]. Bjorklund's group has used PET and ^{11}C-labeled 2β-carbomethoxy-3β-(4-fluorophenyl) tropane ([^{11}C]CFT) to obtain parallel evidence of dopaminergic (DA, associated with Parkinson's disease) cell differentiation *in vivo* [37]. Behavioral recovery of rotational asymmetry at 9 weeks after implantation of ESCs in animal models implicated that ESCs could become a donor source for cell therapy in Parkinson's disease (PD), and the results were shown in Figure 2. Bradbury's group has monitored the long-term viability and proliferation of hESC-derived neural precursor grafts in the brains of immunodeficient and immunocompetent mice using BLI [52]. Their studies demonstrated that there was no significant alteration in the viability of transduced hESC-derived neural precursors in immunodeficient models over a 2-month period, but there were variations in proliferative activity among grafted animals. These studies indicate the broad application prospects of molecular imaging techniques in stem cell therapy for nervous disease.

4. Conclusions and Future Prospects

As we know, stem cells have the following features. First, self-renewal is the hallmark property of stem cells in normal and diseased tissues. Second, the cells that continue to divide over long periods of time are much more likely to accumulate mutations that cause neoplasia and other diseases. Third, in normal tissues that contain self-renewing stem cells, such as the epithelia, the genetic changes which may cause tumorigenesis probably also occur in the stem cells

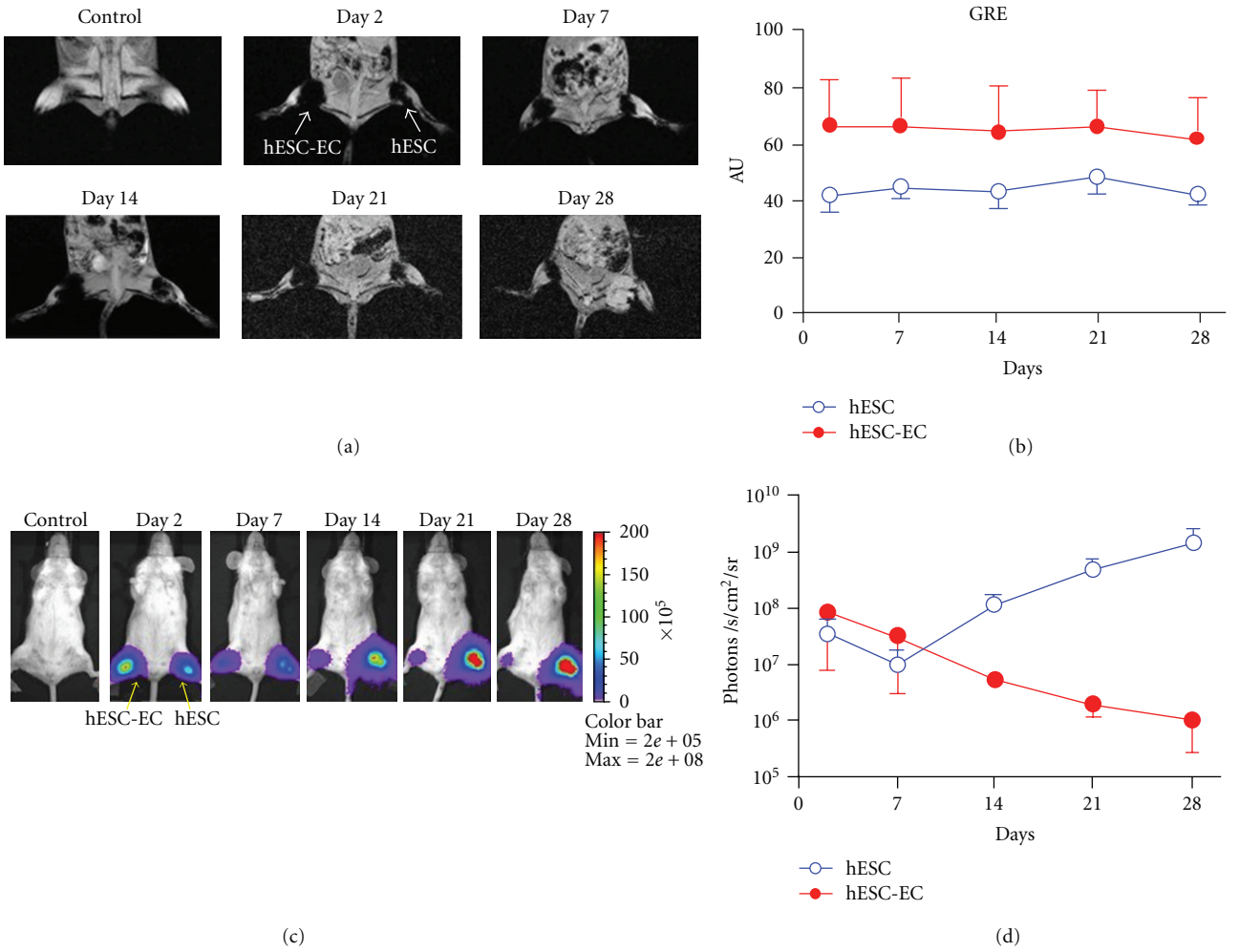
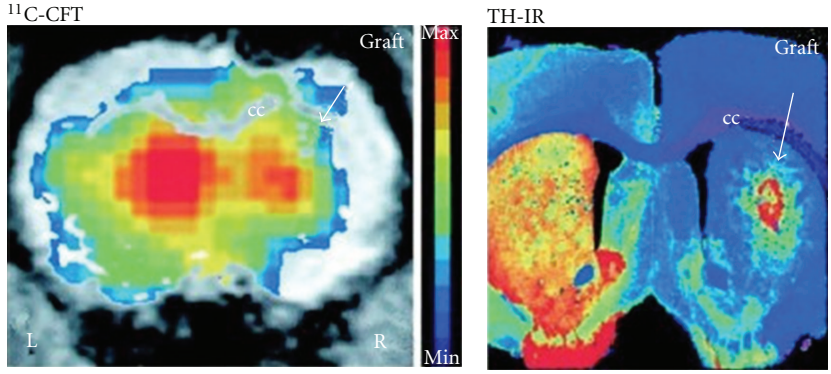


Figure 1: Direct comparison of reporter gene imaging (genetic labeling) versus iron particle imaging (physical labeling) for tracking stem cells. (a) Human ESCs were cultured under normal conditions or on gelatin/fibronection-coated plates to induce endothelial cell differentiation. These predifferentiated human ESC-derived endothelial cells (hESC-ECs) and undifferentiated ESCs were SPIO-labeled (with Feridex) and 1 × 106 cells were then injected into mouse hindlimbs. MR images of one representative animal show the cells at days 2, 7, 14, 21, and 28. (b) MR does not show survival differences between the two groups, as the signal is steady throughout all imaging timepoints, with a higher signal in the hESC-EC group through day 28. (c) These same ESCs were transduced with the human ubiquitin promoter driving fireflyluciferase (Fluc) and enhanced green fluorescence protein (eGFP). These cells were then cultured as in (a) prior to transplantation into the hindlimb of a mouse. (d) BLI showed divergent survival profiles for the two groups, with proliferation of ESC and acute donor cell death of pre-differentiated hESC-ECs. This study demonstrated that MRI provided detailed information on the anatomical location of cells, but not on cell viability. Reporter gene imaging is a better indicator of cell viability and proliferation [36].

or in progeny that acquire the potential for self-renewal [1]. Finally, self-renewing of stem cell is controlled by distinct signaling pathways in different tissues. All the characters of stem cells promote the researches on culture, isolation, clinical application, and other related technologies.

In recent years, many isolation and culture technologies have been proposed, which are reviewed in the above sections. These technologies have promoted the application of the stem cells on research of disease mechanism and clinical therapy. However, most types of stem cells have their own disadvantages for therapeutic applications, such as lack of availability, risk of immune rejection, directional regulation, and ethical controversy. The recent discovery of iPSCs holds the potential to solve these problems. However, the low efficiency of iPSCs generation and their therapeutic safety need to be further studied before initiation of human clinical trials [53, 54]. The speed and efficiency of iPSCs generation from both mouse and human somatic cells can be enhanced by adding vitamin C to the culture medium [55] and the safety of iPSCs may be improved by using nonintegrating viral vectors [56, 57]. Therefore, we expect that clinical application of iPSCs will be achieved in the near future, which will be a historical leap in the medicine area.

To monitor the efficacy of stem cell therapy and develop new isolation and culture technology, it is necessary to improve molecular imaging techniques. For example, new reporter gene systems under development for PET imaging was reported as a new *in vivo* reporter gene imaging system



(a) (b)

Figure 2: The specific dopamine transporter (DAT) ligand [^{11}C] CFT binding in the right grafted striatum was detected using PET, as shown in this brain slice (a). Increased [^{11}C] CFT binding was found in the right striatum, which may correlate with the postmortem presence of TH-immunoreactive (IR) neurons in the graft (b). Color-coded (activity) PET images were overlaid with MRI images for anatomical localization [37].

to monitor transduced ESCs in mice [58, 59]. Meanwhile, Cerenkov imaging technology is under development for its equivalence with PET [60, 61]. Optical molecular imaging systems are also being improved from both reconstruction accuracy and speed. Furthermore, "Opti-PET" instrumentation that will combine advantages of optical imaging with high resolution, low cost, and quantitation ability of PET is also under development [62, 63]. Although none of these imaging technologies fulfill all of the requirements needed for stem cell therapy research at present, their improvement and the development of multimodality molecular imaging system will promote more effectively the understanding of stem cell therapy biology and its mechanisms.

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Research Article

Conditioned Medium from Adipose Tissue-Derived Mesenchymal Stem Cells Induces CD4+FOXP3+ Cells and Increases IL-10 Secretion

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Mesenchymal stem cells (MSCs) are a new and promising tool for therapy of autoimmune disorders. In recent years their possibility to take part in the modulation of the immune response is discussed. The exact mechanisms for immunoregulation realized by MSCs are not clear yet, but interactions with other immunoregulatory cells may be involved in this process. The investigation of the influence of MSCs on the expression of FoxP3 and cytokine secretion by T helper cells was the aim of this study. T helper cells were isolated from PBMCs by magnetic separation and MSCs were isolated from human adipose tissue, and CD4+ T cells were cultured with conditional medium of MSCs. The methods which were used include flow cytometry, ELISA, and Human Proteome profiler kits. The results demonstrated that secretory factors in MSCs conditional medium lead to increased expression of FoxP3 and increased secretion of IL-10 by T helpers. The obtained results give us opportunity to discuss the interaction between two kinds of immunoregulatory cells: MSCs and FoxP3+ T helpers. We suppose that this interaction leads to increased number of immunosuppressive helpers which secrete IL-10. MSCs provide some of their immunosuppressive functions acting on T regulatory cells, and we believe that IL-6 secreted by MSCs is involved in this process.

1. Introduction

The process of immunomodulation is of utmost importance for sustaining the immune homeostasis in the organism, and detailed knowledge about the precise mechanisms of this process can bring about a better and more efficient therapy of the autoimmune diseases. Regulation of the immune response is accomplished by a number of cell subtypes and secreted factors as a lot of attention in the last years is focused on the role of the mesenchymal stem cells (MSCs) [1–3]. Numerous papers have reported data proving the important immunoregulatory functions of the human MSCs. It has been established that MSCs inhibit the proliferation of T and B cells, the production of H_2O_2 from neutrophils, the secretion of immunoglobulins, T and NK cytotoxicity as well as the differentiation and maturation of monocytes into dendritic cells. Also, the immunosuppression caused by the MSCs affects the secretion of cytokines which is biased to Th2 dominance with secretion of IL-10 and IL-4 while the secretion of IFNγ and TNFα is suppressed [3–7]. The precise mechanisms of the MSCs immunosuppressive effect have not been clarified yet. Specific roles of secreted

factors such as IDO, IL-6, TGFβ, LIF, INOs, PGE2, HLA-G [3, 5–8] have been assigned, and the role of direct cell-to-cell contact between the MSCs and the targeted cells is discussed [9, 10]. However, besides data about the direct inhibitory effects of MSCs, there are data published on some indirect inhibitory effects which are mediated through influencing other immunoregulatory cells. Previous studies in our laboratory have demonstrated that MSCs inhibit the differentiation of monocytes to dendritic cells and down-regulate the expression of molecules related with the antigen presentation and the expression of CCL-3 and CCL-4. These experiments showed that the effect of MSCs isolated from adipose tissue (AT-MSCs) is stronger in comparison to MSCs isolated from human bone marrow (BM-MSCs) [11]. Some indirect immunosuppressive effects of MSCs seem to be due to their influence on the classical immunoregulatory CD4+FoxP3+ cells generally referred to as Tregs. The last decade witnessed some significant development of the idea for the T regulatory cells. Along with the Th3 cells secreting TGFβ and Tr1 secreting IL-10 [12, 13] a role was established for CD4+CD25+ T lymphocytes which exert immune suppression via CTLA-4, TGFβ, and/or IL-10 [14–17]. Later on an intracellular transcription factor FoxP3 was identified as a more precise marker for the T regulatory subpopulations [18–20]. It has been established that about 2% of CD4+ cells express FoxP3 [18], and it is related to the differentiation of Tregs and exerts direct functional immune suppression [13, 17, 21]. Recently, the role of CD25 molecule as a marker for suppressive subpopulations has raised some doubts. It is clear that the lack of surface expression of CD127 more accurately correlates with FoxP3, and this can be found even in CD25 negative cells [18, 22, 23]. Even more in patients with systemic lupus erythematosus (SLE) both CD4+CD25+FoxP3+ and CD4+CD25−FoxP3+ cells with expressed immunosuppressive properties have been identified [24, 25]. That is the reason Tregs are more often designated as CD4+FoxP3+. The molecular mechanisms of immunosuppression exerted by CD4+FoxP3+ cells have not been identified but it is quite obvious that these mechanisms include the secretion of TGFβ and/or IL-10 [12, 17–19].

Some published data seem to show that under the influence of MSCs the number of the CD4+FoxP3+ cells is increased, and these cells produce the immunosuppressive cytokine IL-10, and thus MSCs exert indirect immunosuppressive effect [2, 7, 9, 10, 26].

The aim of the present study is to analyze the effect of conditioned medium of AT-MSCs on the expression of FoxP3 molecule and the secretion of cytokines by a homogeneously purified subpopulation of CD4+ T lymphocytes. Additionally the factors secreted from the AT-MSCs are analyzed with the view of their putative effect on the changes in the CD4+ T lymphocyte populations.

2. Materials and Methods

2.1. Materials. Samples of peripheral blood were collected by venepuncture from 12 healthy volunteers, and 12 samples of human adipose tissues were collected during hip surgery. In both cases the samples were taken after a signed informed consent from the donors according to the regulations in this country.

2.2. Mesenchymal Stem Cells. Mesenchymal stem cells from adipose tissue were isolated, cultured, and phenotyped following strictly the generally accepted laboratory protocols and most of all the protocol developed in our laboratory [4].

2.3. Conditioned Medium. After forming a monolayer the AT-MSCs were detached after treatment with Trypsin EDTA (1 : 250) (PAA, Austria) and seeded in 6-well plate (passage 1) at concentration 3–5 $\times$ 10^4 cell/well in DMEM medium supplement with 10% foetal bovine serum and antibiotics (PAA, Austria). The cells were cultured until reaching 80% confluency as the culture medium was changed every 48 hours. After the last change of the medium, the 80% confluent cells were further cultured for 48 hours, and the medium designated as "conditioned AT-MSC medium" was collected. Thus the conditioned AT-MSC medium contained DMEM, FBS, and any factors secreted by the nonactivated AT-MSCs. This procedure was strictly followed in 12 independent experiments performed with T cell isolated and purified from 12 individual donors and 12 different samples of conditioned medium obtained as described above.

2.4. Isolation of T Helper Lymphocytes. PBMCs (peripheral blood monocytic cells) were isolated from peripheral blood by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare). The isolated cells were used for purification of CD4+ T lymphocytes using magnetic separation with MACS kits (Miltenyi Biotec, Bergisch-Gladbach, Germany). Initially, CD14+ monocytes were discarded from PBMCs by positive selection with anti-CD14-microbeads and the flow through fraction was collected. CD4+ cells were enriched from the resulting CD14− cell fraction using anti-CD4-microbeads and the enriched CD4+ cell fraction was further characterized by flow cytometry mostly with regard to the expression of CD3/CD4 markers.

2.5. Cell Cultures. The isolated T helpers (Th) were cultured for 48 hours in conditioned medium from AT-MSCs at the first passage cultured for 48 hours. Control T helper cells were cultured in DMEM low glucose medium supplemented with 10% fetal bovine serum (PAA, Austria). Th cells were seeded at concentration 5 $\times$ 10^6 cells/well into 24-well plates (PAA, Austria).

2.6. Flow Cytometry. Homogeneity of the purified T helper populations was proved using monoclonal antibodies anti-human CD3 FITC and anti-human CD4 PE. Anti-human CD4 FITC, anti-human HLA-DR PE, and anti-human CD69 PE antibodies were used to assess the surface expression of the activation markers by CD4+ T lymphocytes cultured in AT-MSCs conditioned medium as well as the control T helper cultures. Identification of T helper cells expressing surface CD25 and intracellular FoxP3 was performed using CD4 PerCP-Cy 5.5, anti-human CD25 FITC, anti-human

FoxP3 PE and FoxP3 Buffer set following the standard procedure recommended by the producer company. All the antibodies and the machines (FACSCalibur) used were purchased from Becton Dickinson, CA, USA.

2.7. Enzyme Linked Immunosorbent Assay (ELISA). Supernatants from Th lymphocytes cultured with AT-MSCs conditioned medium or from control Th cell cultures as well as samples of AT-MSCs conditioned medium were tested for the presence of IL-2, IFNγ, IL-10, and TGFb by Human Instant ELISA (Bender MedSystems, Austria) following strictly the manufacturer's instructions.

2.8. Proteome Profiler Kits. Analysis of the cytokines in the AT-MSCs culture supernatants was performed using the Human Cytokine array panel A array kit (R&D Systems, MN, USA) which can detect 36 different cytokines, chemokines, and growth factors (C5a, CD40L, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNFα, TREM-1).

With the aim to assess any signs of apoptosis of the CD4+ T cells cultured in AT-MSCs conditioned medium or CD4+ control cells the Proteome profiler human apoptosis kit (R&D Systems, MN, USA) was used as this test is capable to establish the intracellular expression of Bad, Bax, Bcl-2, Bcl-x, Pro-Caspase-3, Cleaved Caspase-3, Catalase, clap-1, clap-2, Claspin, Clusterin, Cytochrome C, TRAIL R1/DR4, TRAIR R1/DR5, FADD, Fas/TNFSF6, HIF-1α, HO-1/HMOX1/HPS32, HO-2/HMOX2, HSP27, HSP60, HSP70, HTRA2/Omi, Livin, PON2, p21/CIP1/CDNK1A, p27Kip1, Phospho-p53(S15), Phospho-p53(S46), Phospho-p53(S392), Phospho-RAD17(S635), SMAC/Diablo, Survivin, TNF RI/TNFRSF1A, XIAP.

2.9. Software. The programs CellQuest and WinMDI 2.9 were used for analysis of the flow cytometry data. Image J program (NIH, Bethesda, MD, USA) was used for analysis of the results from the Human Cytokine array panel A array kit and Proteome profiler human apoptosis kit.

2.10. Statistics. Statistical analyses of cytokine concentrations were performed using paired Student's t-test. Data were presented as mean ± standard deviation (SD). Statistical significance was established at $P < 0.05$.

3. Results

3.1. Secreted Factors Present in AT-MSCs Conditioned Medium Cause an Increase of the Numbers of T Helper Cells Expressing FoxP3. The procedure followed in our experiments leads to isolation of a very homogeneous population of CD3+CD4+ T helper lymphocytes (Figures 1(a) and 1(b)). Cultivation of these cells in AT-MSCs conditioned medium did not cause any alterations in the expression of the activation markers CD69 and HLA-DR (data not shown). However, cultures of Th cells in conditioned medium caused an increase of the numbers of CD4+ T lymphocytes expressing FoxP3 (Figure 1(d)) as compared to the same parameter in control cells (Figure 1(c)). This effect was found for both T helpers positive for the surface expression of CD25 and for T helpers negative for CD25 and was recorded in all samples tested from individual donors (Table 1). So, it is obvious that some secreted factors present in AT-MSCs conditioned medium induce a specific upregulation of the transcription factor FoxP3 which defines the Treg subpopulation regardless of the expression of CD25.

3.2. Secreted Factors Present in AT-MSCs Conditioned Medium Caused an Upregulation of the IL-10 Secretion by T Helper Lymphocytes. The search of secreted cytokines revealed the presence of TGFβ in very low concentrations in both CD4+ T cells cultured in AT-MSCs conditioned medium and control CD4+ T medium samples in all cases tested (Figure 2(a)). No statistically significant differences were found for the concentrations of IFNγ or IL-2 in experimental or control samples in each experiment with donor lymphocytes (Figures 2(b) and 2(c)). In contrast to these findings statistically significant higher concentration of IL-10 was detected in supernatants from CD4+ T cells cultured in AT-MSCs conditioned medium in comparison to the control supernatants (Figure 2(d)). This increased secretion of IL-10 was recorded in all cases of Th cells cultured in AT-MSCs conditioned medium. It should be pointed out that the AT-MSCs conditioned medium itself did not contain any IL-10, and this gives us ground to conclude that the increased concentration of this cytokine by T helpers cultured in AT-MSCs conditioned medium is due to its secretion by activated Th lymphocytes.

3.3. Secreted Factors in AT-MSCs Conditioned Medium Did Not Induce any Changes in the Pro- and Antiapoptotic Factors in T Helper Lymphocytes. One of the possible ways for MSCs to alter the T helper cells is the influence of the process of apoptosis, and in order to check this possibility the presence and changes of the intracellular proapoptotic or antiapoptotic factors were assayed in the same experimental design. However, no significant differences were found between Th cells cultured in AT-MSCs medium and the control cells (data not shown).

3.4. AT-MSCs Conditioned Medium Contains Some Chemokines and IL-6. Repeated testing of the AT-MSCs conditioned medium showed that it contained several chemokines with known effect on T helper cells and just a single cytokine with proven immunoregulatory effect. The chemokines were Groα, RANTES, Serpin E, IL-8, and SDF-1 and from the cytokines the IL-6 was found to be with quite high concentration in the AT-MSCs conditioned medium. These chemokines and IL-6 were found in negligible concentrations in control culture medium which point to their secretion by AT-MSCs (Figure 3).

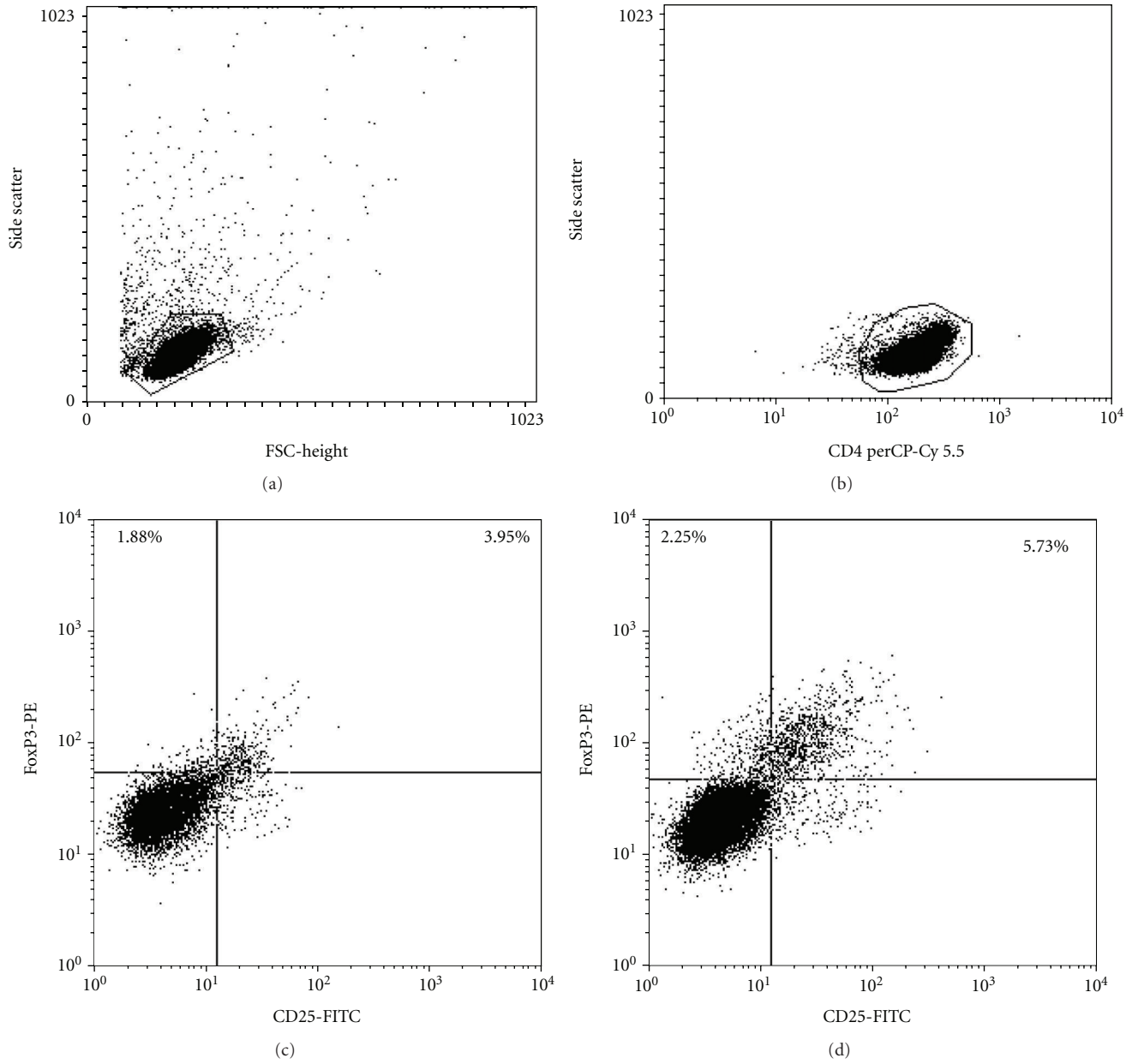

Figure 1: A representative dot plot showing the expression of CD25 and FoxP3 markers by T helpers. Panels (a) and (b) show the homogeneity of the purified CD4+ population; (c) shows the percentage of FoxP3+ cells in control culture; (d) shows an increase of the percentage of cells expressing FoxP3 after being cultured in AT-MSCs conditioned medium.

4. Discussion

The results obtained showed that when homogeneous population of CD4+ T lymphocytes was cultured in AT-MSCs conditioned medium, the percentage of the T helpers expressing FoxP3 is increased as both CD25+ and CD25− cell subpopulations were affected. The role of the CD4+CD25+FoxP3+ cells as the major immunosuppressive factor has been known for a rather long time. It has been described that these cells can originate directly from the thymus and are designated as natural (nTregs) or can differentiate under the influence of cytokines in the periphery and are called induced (iTregs) [13, 19, 27]. The major cytokines responsible for inducing the differentiation of iTregs are most probably IL-2 and TGFβ [28]. We could not detect these cytokines in the AT-MSCs conditioned medium, but judging on the increased percentage of CD4+CD25+FoxP3+ it can be speculated that other cytokines may be involved in this process as discussed in the following.

Further on, our results revealed that under the influence of AT-MSCs conditioned medium, the percentages of the recently described subpopulation CD4+CD25−FoxP3+ increase as well. These cells were found to be increased in SLE patients and were considered to be progenitors of CD25+

Table 1: The effect of AT-MSCs conditioned medium on the percentage values of CD4+FoxP3+ T lymphocytes in cultures of T helper cells obtained from different donors. The data from all subjects tested revealed the stable tendency towards an increase of CD4+CD25+FoxP3+ and CD4+CD25−FoxP3+ cell numbers in the presence of AT-MSCs conditioned medium compared with the respective control cultures.

	CD4+CD25+FoxP3+		CD4+CD25−FoxP3+	
	w/o AT-MSCs c.m.	With AT-MSCs c.m.	w/o AT-MSCs c.m.	With AT-MSCs c.m.
Donor 1	3.45%	4.42%	2.12%	4.12%
Donor 2	2.19%	2.91%	1.72%	2.37%
Donor 3	5.04%	5.52%	1.74%	2.26%
Donor 4	1.47%	2.48%	2.84%	3.06%
Donor 5	3.95%	5.73%	1.88%	2.25%
Donor 6	2.87%	4.55%	1.01%	1.95%
Donor 7	5.80%	7.65%	2.84%	4.32%
Donor 8	5.69%	5.81%	3.13%	3.78%
Donor 9	1.30%	3.33%	1.41%	2.32%
Donor 10	1.77%	2.03%	0.7%	1.10%
Donor 11	1.90%	5.39%	0.7%	0.92%
Donor 12	2.92%	5.06%	1.6%	1.7%

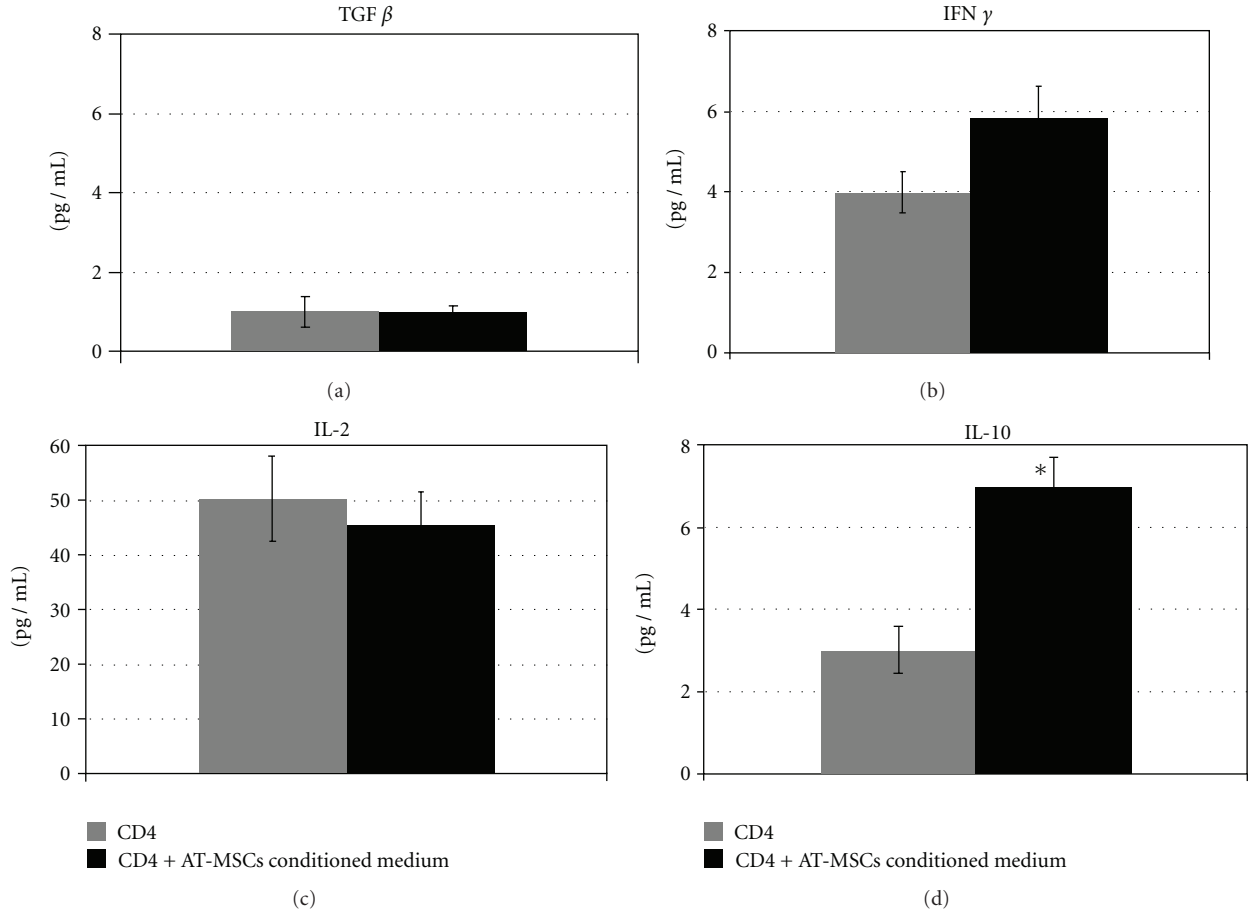

Figure 2: Cytokine secretion by CD4+ T lymphocytes cultured in AT-MSCs conditioned medium or control DMEM medium. Cultivation of CD4+ T cells in AT-MSCs conditioned medium did not induce changes in the secretion of TGFβ (a), IFNγ (b), or IL-2 (c). In contrast the secretion of IL-10 was statistically significantly upregulated ($^*P < 0.05$) in the presence of AT-MSCs conditioned medium compared with the control (d). Mean values ± SD of 12 independent experiments with cells from different donors are shown.

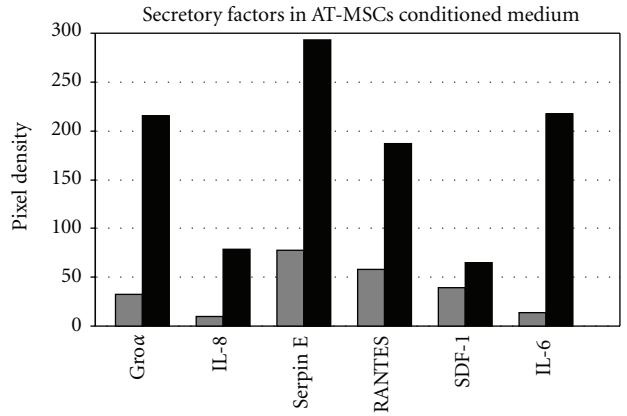

Figure 3: Cytokines and chemokines found to be present at significant levels in AT-MSCs conditioned medium (black columns) compared to control DMEM medium (grey columns). The figure represents only the cytokines and chemokines which are known to affect T helper activities.

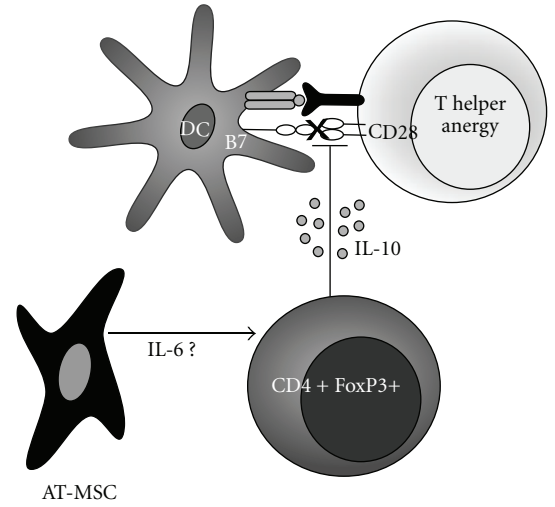

Figure 4: A possible indirect mechanism of the immunoregulatory effects of factors secreted by AT-MSCs.

cells [24]. Different opinions are discussed in the specialized literature as some authors expressed some doubts whether this subpopulation is immunoregulatory by itself [18], but the prevailing opinion is independently to the expression of CD25, the FoxP3 by itself exert functional suppression [13, 17, 21, 29]. Based on this assumption the regulatory cells are more often designated as CD4+FoxP3+ [18, 19]. It is commonly accepted that the CD4+FoxP3+ cells express their immunosuppressive effects through the secretion of TGFβ and/or IL-10 [12, 15, 17, 18]. In our studies we did not record an increased secretion of TGFβ by CD4+ T lymphocytes cultured in AT-MSCs medium. It is quite possible that under experimental design used in our experiments this cytokine is being expressed as membrane-bound form at the surface of the CD4+ T lymphocytes as such a mechanism has been described for CD4+FoxP3+ cells and still is able to express its suppressive effect [14, 19]. However, statistically significant increase was found for IL-10 secreted by CD4+ T lymphocytes cultured in AT-MSCs conditioned medium. The secretion of IL-10 by CD4+FoxP3+ cells is considered to be one of the most important mechanisms via which this cell subpopulation exerts the immunosuppressive function [30, 31, 31]. IL-10 is an anti-inflammatory cytokine which inhibits the secretion of proinflammatory cytokines by the macrophages and dendritic cells [30]. It inhibits the activities of Th17 [31] and the differentiation of blood monocytes into dendritic cells [11, 30]. One of the most significant immunoregulatory effects is the induction of tolerance in dendritic cells by inhibition of molecules which are crucial for antigenic presentation such as the B7 complex [11]. Moreover, IL-10 directly inhibits the CD28 expression on Th surface, and this molecule is the specific ligand for the B7 complex. Our previous results have shown that AT-MSCs upregulate the secretion of IL-10 by monocytic dendritic cells [11]. However, under the experimental conditions described it cannot be claimed that the CD4+FoxP3+ cells are the only source of the IL-10, but having in mind the increased number of these cells it is quite possible that there is a causative link between the increased numbers of CD4+FoxP3+ cells and the increased concentrations of IL-10 in the culture medium. It can be further speculated that under the influence of the AT-MSCs conditioned medium the number of CD4+FoxP3+ cells secreting IL-10 increases, and this could lead to an inhibition of the costimulatory molecule expression on the surface of the dendritic cells and T helpers which is the most important condition for inducing anergy in the T helper cells. Therefore, together with the other mechanisms for immune suppression the AT-MSCs realize an indirect immune suppression by influencing other immunomodulatory cells such as dendritic cells and CD4+FoxP3+ lymphocytes (Figure 4). AT-MSCs induce secretion of IL-10 by CD4+FoxP3+ T cells. This cytokine inhibits the expression of key molecules of antigen presentation, and this process leads to anergy of the T lymphocytes. It can be speculated that IL-6 secreted by AT-MSCs is one of the key elements responsible for this effect.

It is known that in parallel to its anti-inflammatory effect the IL-10 has proapoptotic effect on dendritic cells as shown in a previous paper [11]. The present studies did not find any changes with regard to the expression of the intracellular pro- or antiapoptotic factors in CD4+ T lymphocytes cultured in the presence of AT-MSCs conditioned medium. It has been reported that the MSCs in general realize their immunomodulatory functions either through direct contact between the cells [6] or through secretion of cytokines.

As mentioned above AT-MSCs conditioned medium was used in our experiments which means that the observed effects are due to the presence of secreted factors in the medium. Chemokines such as Groα, RANTES, Serpin E, IL-8, and SDF-1 which influence the chemotaxis of the T lymphocytes were shown to be present in the conditioned medium samples. So, it can be assumed that the process of immunosuppression realized by the AT-MSCs would be

initiated with recruiting the T lymphocytes as the first step of the process.

Several types of secretory factors secreted by MSCs have been reported in the literature which can influence the formation of CD4+FoxP3+ cells, and these include IDO, TGFβ, INOs, PGE2, HLA-G5, I-309, IL-10, IL-6 [6–9, 31].

In our experiments the presence of IL-6 solely was recorded in samples of AT-MSCs conditioned medium although we searched for TGFβ, I-309, and IL-10, too. A probable explanation of this fact would be the assumption that MSCs secrete a number of cytokines after being stimulated by other cytokines [3]. Thus, after stimulation with IFNγ the MSCs secrete I-309 which on its side induces the formation of Tregs [8] while the direct contact and IL-10 secretion have a key role in induction of the secretion of HLA-G5 which would have the same effect [9]. Our results show that such an induction is not needed for the secretion of IL-6 by AT-MSCs. The generally accepted views for IL-6 hold that it is a proinflammatory cytokine which has a role in inhibition of the Tregs and stimulation of the Th17 immune response [18, 32]. However other data would suppose that the function of IL-6 should be reassessed because of its reported immunosuppressive activity. IL-6 causes increased secretion of IL-10 and can exert antiapoptotic activity [7, 33]. It has been reported that MSCs secrete high levels of IL-6, and this secretion correlates directly with inhibition of the T cells, inhibition of the differentiation of the dendritic cells, and inhibition of the secretion of proinflammatory cytokines as well [34]. Similarly, the IL-6 increases the secretion of other immunosuppressive factors such as INOs and PGE2 by MSCs acting by an autocrine mechanism [35] and also directly causes an increase of the numbers of newly described CD8+FoxP3+ immunoregulatory cells [29, 36]. The numerous activities of the IL-6 mentioned give ground to discuss its role as an anti-inflammatory cytokine. In agreement with the above cited data and our own results it can be speculated that under the influence of the IL-6 secreted by nonstimulated AT-MSCs the number of IL-10 secreting CD4+FoxP3+ cells increases, and thus AT-MSCs realize an indirect immunosuppressive effect via the Tregs. Really it should not be claimed that the IL-6 is the only one cytokine in this process since most of the factors modulating CD4+FoxP3+ cell functions have not been tested in our experiments. Most probably the cytokine induction of MSCs to secrete immunosuppressive factors as well as the direct cell-to-cell contacts with the subsequent secretion of HLA-G5 would be of high importance. Previous results from our laboratory have shown the presence of HLA-G5 in the cytoplasm of AT-MSCs [37] as the effect of this molecule with regard to the Tregs cells has been described in several papers [1, 5–7].

5. Conclusion

In conclusion, the results obtained show that under the influence of factors secreted by AT-MSCs the numbers of CD4+FoxP3+ lymphocytes are increased and the secretion of IL-10 is upregulated in purified CD4+ T cell population. It can be supposed that the presence of IL-6 is very important for this process. Similar effects of MSCs on the FoxP3+ cells and secretion of IL-10 have been described in other papers [2, 6–9] but bone-marrow-derived MSCs and umbilical cord-derived MSCs have been used, and they were cocultured with heterogeneous cell mixture of PBMCs. Original features in our experiments are the use of AT-MSCs conditioned medium and a homogeneous population of CD4+ T lymphocytes. Under these conditions there is no direct cell-to-cell contact which might activate the AT-MSCs by other cell types, and the "native" or "passive" secretion of cytokines by AT-MSCs and their effects on the purified T helper cells were observed.

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Review Article

Bone Morphogenetic Proteins in Craniofacial Surgery: Current Techniques, Clinical Experiences, and the Future of Personalized Stem Cell Therapy

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Critical-size osseous defects cannot heal without surgical intervention and can pose a significant challenge to craniofacial reconstruction. Autologous bone grafting is the gold standard for repair but is limited by a donor site morbidity and a potentially inadequate supply of autologous bone. Alternatives to autologous bone grafting include the use of alloplastic and allogenic materials, mesenchymal stem cells, and bone morphogenetic proteins. Bone morphogenetic proteins (BMPs) are essential mediators of bone formation involved in the regulation of differentiation of osteoprogenitor cells into osteoblasts. Here we focus on the use of BMPs in experimental models of craniofacial surgery and clinical applications of BMPs in the reconstruction of the cranial vault, palate, and mandible and suggest a model for the use of BMPs in personalized stem cell therapies.

1. Introduction

Critical-size osseous defects cannot heal without surgical intervention and pose a significant challenge to craniofacial reconstruction following infection, trauma, tumor, or congenital disease. Autologous bone remains the current gold standard source of donor tissue for the surgical repair of critical-size craniofacial defects; however, reconstruction of these defects is often limited by a potentially inadequate supply of bone for autograft [1–3]. Autografts from the iliac crest, ribs, tibia, or other sites are limited in supply and can incur significant donor site morbidity in 8–10% of patients [2, 4–13]. Calvarial bone grafting is considered the gold standard for repair of craniofacial defects, and data both from experimental animals and clinical experiences have demonstrated that intramembranous bone grafts such as those from the calvarium undergo less resorption than grafts from endochondral bone sites do [14]. Furthermore, the harvest of calvarial split-thickness bone grafts is associated with minimal donor site morbidity [7, 11–14].

Craniofacial reconstruction may be even more difficult in the pediatric patient because sources of bone for autograft are especially limited in children. Prior to two years of age, a child's dura mater has significant capacity for reossification of the calvarium; after two years of age, it loses its propensity to induce significant reossification of overlying bone defects [15–17]. In addition, in the case of pediatric patients, calvarial bone grafting is not a viable option until the child is six to ten years old, when the skull is developed enough to tolerate split-thickness bone grafting [2, 3, 15–19]. The most common morbid complication of autograft is bone pain at the donor site, but these complications can also potentially include hematoma, infection, chronic pain, nerve damage, and unanticipated fracture [1, 5, 6, 18].

Allogenic implants such as demineralized or cadaveric bone and alloplastic bone substitute materials such as hydroxyapatite cement, methyl methacrylate, ceramics, titanium, and porous polyethylene have also been used for the reconstruction of craniofacial defects [30, 31]. Reconstruction of bony defects with alloplastic substances has been

shown to facilitate osseous healing in a wide variety of orthopedic, neurosurgical, and craniofacial surgical scenarios by facilitating the migration of bone-forming cells into different types of scaffolds [32, 33]. However, several complications and adverse outcomes are feared and have been reported with the use of alloplastic and allogenic bone substitutes. These risks include graft infection, induction of an immune response, and transmission of infectious disease [32, 33]. For example, Wong et al. found that 59% of pediatric patients treated with hydroxyapatite cement for craniofacial repairs ultimately had infectious complications within the following year [34]. A major drawback of these agents is their inability to mediate osteoinduction and their failure to incorporate into surrounding normal bone (osseointegration). Thus, further evaluation of the safety and efficacy of these materials is warranted.

Alloplastic materials have been used alone and in conjunction with osteoprogenitor cells, as well as with bone morphogenetic proteins (BMPs) to facilitate bone regeneration [32]. BMPs have been proven to be important mediators of bone formation involved in the regulation of differentiation of osteoprogenitor cells into osteoblasts [35–43]. BMPs can be used to drive *in vitro, in vivo,* and *ex vivo* differentiation of adult-derived osteoprogenitor cells into bone-forming osteoblasts, and investigation into different conditions of BMP stimulation may provide insight into the means of achieving optimally effective bone tissue generation [43].

While BMPs hold great promise for craniofacial reconstruction, significant concern has been generated over the safety of the currently commercially available forms of recombinant human BMPs (rhBMPs) rhBMP-7 and rhBMP-2 due to reports of clinically significant operative site edema in craniomaxillofacial and spinal applications [29, 44–50]. The effects of direct application of exogenous BMPs to bone defects may also prove to be too unpredictable for clinical use. Certain studies in experimental animals have shown that BMPs may actually inhibit bone formation *in vivo,* and the application of rhBMP to the repair of the human spinal column has been shown to be associated with resorption of vertebral bodies in certain cases [51, 52]. Thus, future therapies involving the implantation of osteoblastic cells differentiated *ex vivo* from mesenchymal stem cells by exposure to BMPs may prove to be the safest and most efficacious mode of therapy.

2. Bone Morphogenetic Proteins (BMPs)

Bone morphogenetic proteins belong to the transforming growth factor β (TGF-β) superfamily of structurally related dimeric disulfide-linked receptor ligands [53–57]. The TGF-β superfamily includes proteins involved in the regulation of organismal development, cell differentiation, cell growth, and cell proliferation [55]. They are expressed by most cells in the body, and alterations in expression are implicated not only in normal biological processes such as organogenesis, embryogenesis, fracture healing, and wound healing, but also in pathological states including cancer, atherosclerosis, fibrotic diseases, and developmental diseases [54, 58].

Cranial neural crest cells are responsible for the formation of the craniofacial skeleton via intramembranous ossification, the process whereby mesenchymal cells directly differentiate into osteoblasts to form intramembranous flat bones. Intramembranous ossification occurs without the intermediate development of a cartilaginous scaffold seen in endochondral ossification of the axial and limb skeletons. The axial and limb skeletons are embryonically derived from paraxial mesoderm and lateral plate mesoderm cells, respectively, in contradistinction to the derivation of the craniofacial skeleton from neural crest cells. The osteogenic differentiation of craniofacial neural crest cells in normal development relies on signaling interactions with the overlying epithelium and includes a large number of extracellular matrix molecules, transcription factors, and cytokines including bone morphogenetic proteins [59, 60]. The diversity of function and expression of bone morphogenetic proteins and other growth factors may be responsible for the differences in architecture and morphogenesis of bone at different locations in the vertebrate skeleton [61].

Bone morphogenetic proteins were first identified as proteins capable of inducing de novo cartilage and bone formation *in vivo* following subcutaneous implantation [61–65]. Initially the formation of ectopic bone in early experimental models was ascribed to unknown properties of demineralized bone. Later these results were shown to have been caused by the presence of BMPs in the demineralized bone matrix [62, 66]. BMPs have since been shown to play many other roles in vertebrate development, including morphogenic signaling in gastrulation, patterning of the vertebrate embryonic body plan along the dorsal-ventral axis, limb patterning and development, and development of the nervous system, kidneys, lungs, heart, gut, teeth, skin, and gonads [59, 67].

3. Mechanism of Bone Morphogenetic Protein Induction of Osteogenesis

Ligand-receptor interaction in the TGF-β superfamily is initiated by the formation of a heterodimeric serine-threonine kinase receptor complex [68–73]. BMP signaling is transduced primarily by interaction with the BMP type I receptors ALK-2, -3, -4, and ALK-6 [74, 75] (Figure 1). The close proximity of the constitutively active type II receptor kinase to the type I receptor in the heterodimeric BMP receptor-ligand complex permits phosphorylation and activation of the type I receptor kinase, resulting in an active complex that initiates signal transduction in the cell [68, 71]. The ligand-receptor interaction leads to the downstream activation of at least two distinct signal transduction pathways: the canonical Smad-mediated pathway and the noncanonical p38 mitogen-activated protein kinase pathway (MAPK) [76–79].

In the canonical Smad-mediated pathway, the activated type I receptor kinase phosphorylates receptor regulated Smad proteins (R-Smads) [68, 80]. The interaction of R-Smads with membrane-bound BMP receptors is highly specific, and the R-Smads 1, 5, and 8 are recognized and phosphorylated specifically by BMP-activated ligand-Ser/Thr kinase receptor complexes [71]. The phosphorylation of

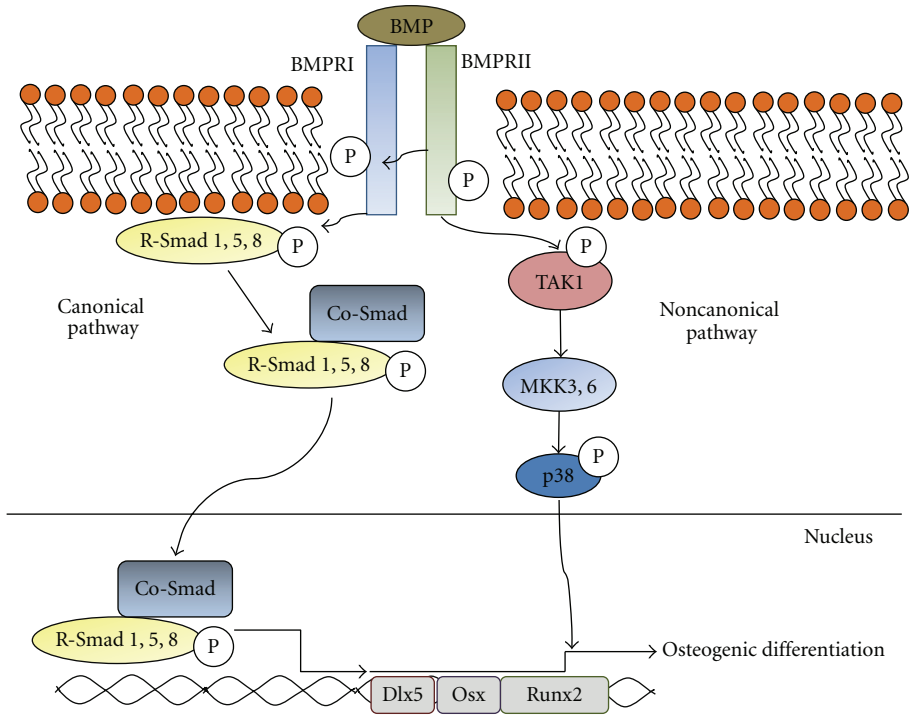

Figure 1: The canonical Smad-mediated and Smad-independent p38 MAPK pathways for BMP signal transduction are shown. In the Smad-mediated pathway, the activation of the BMP receptor complex by BMP ligand interaction leads to phosphorylation of R-Smads 1, 5, and 8, freeing them from the internal surface of the cell membrane. This enables the interaction between R-Smads and Co-Smad proteins, subsequently resulting in exposure of nuclear import sequences that permit the heteromeric complexes to enter the nucleus and activate transcription of osteogenic genes Dlx5, Osterix, and Runx2. In the Smad-independent pathway phosphorylation of TAK1 by the BMP ligand-receptor complex leads to signal transduction through the p38 MAP kinase pathway, resulting in transcription of Runx2.

R-Smads results in the destabilization of R-Smad protein complexes at the inner surface of the cell membrane, releasing R-Smads into the cytoplasm [74, 80, 81]. The release of R-Smads into the cytoplasm permits their binding to the common-partner Smad (Co-Smad) Smad 4 [71, 82]. Smad 4 is the only Co-Smad yet identified in mammalian species and is used for signal transduction by all members of the TGF-β family; thus, the binding of different R-Smads to Smad 4 is critical for determining the specificity of the different signal transduction pathways in the TGF-β superfamily [74]. R-Smads and Smad 4 can act as transcription factors independently or when complexed as heteromers; however, Smads have the greatest effect on transcriptional regulation when R-Smads are bound to Smad 4 as heteromeric complexes [68, 71, 83]. The interaction between the R-Smad and Co-Smad proteins maximally exposes the nuclear import sequences present on the R-Smad proteins, leading to increased sequestration of Smad heteromers in the nucleus [53, 71]. Thus, the BMP ligand-receptor signal cascade permits binding of activated R-Smads 1, 5, or 8 to Smad 4, resulting in the formation of heteromeric R-Smad-Smad4 complexes that are subsequently transported to the nucleus [69, 70, 83].

Once in the nucleus, Smad heteromers are involved in the regulation of osteogenic gene transcription, most importantly through the transcription of the osteogenic master gene Runx2 and its transcriptional coactivators Dlx5 and Osterix [84–86]. BMP signal transduction increases differentiation-stage appropriate expression levels of major markers of osteoblastic differentiation, including the early marker CTGF, early to middle-stage markers alkaline phosphatase (ALP) and Runx2, and late-stage markers Osteopontin and Osteocalcin [39, 87–90]. The transcription factors Osterix, Dlx5, and Msx2 are critically involved in the mediation of the osteogenic effects of BMPs, and the homozygous knock-out conditions of Osterix, Msx2, and Dlx5 in mice have all resulted in significant impairment of osteogenesis [86]. While these transcription factors genes are downstream targets of Runx2 signal transduction pathways and share many of the same downstream activation effects as Runx2, they also can activate different osteogenic genes independently from Runx2 [86]. Runx2 (also known as AML3, CBfa1, and PEBP2A1) remains the critical final master gene target of both TGF-β and bone morphogenetic protein signal transduction pathways [78, 84]. Runx2 induces osteoblastic differentiation and is required for endochondral ossification, intramembranous ossification, and tooth development [77, 91]. Homozygous deletion of Runx2 in mice completely inhibits ossification and is fatal immediately after birth [92, 93]. Heterozygous mutations of Runx2 are thought to produce some cases of the autosomal dominant disease cleidocranial dysplasia, a disease characterized by numerous skeletal abnormalities [79]. Runx2 is essential for the generation of an osteoblastic phenotype, as evidenced by the

failure of Runx2-deficient calvarial cells to differentiate into osteoblasts when stimulated with BMP-2, with stimulation instead causing the generation of hypertrophic chondrocytes [94].

Runx2 is also a final target of the Smad-independent BMP and TGF-β signal transduction pathways [84]. TGF-β activation kinase (TAK1) is also phosphorylated and activated by the Ser/Thr kinase type I receptor subunit, initiating a kinase cascade acting through MKK3 and MKK6, mediating the phosphorylation and activation of p38 mitogen-activated protein kinase [85]. The activation of p38 MAPK ultimately results in transcription of Runx2, a point of convergence for the canonical Smad-dependent pathway and the noncanonical Smad-independent pathway in both TGF-β and BMP signaling [84, 85].

BMP-2-induced expression of osteogenic differentiation markers has further been shown to be mediated differentially not only by the p38 MAP kinase system but also by extracellular signal regulated kinase (ERK) signal transduction, thus indicating that BMP signal transduction is not limited solely to the Smad-dependent and p38 MAPK signal transduction pathways [84, 95]. Further studies have also demonstrated that there is significant crosstalk between the signal transduction pathways of the BMPs and the signaling pathways of Wnt/β-catenin and IGF-1, known inducers of osteogenesis [87]. Interaction with the Wnt/β-catenin pathway in particular influences osteogenesis via the RANK/OPG axis by increasing expression of osteoprotegerin and decreasing expression of RANKL, resulting in decreased osteoclastogenesis and increased bone formation [96].

4. BMP Expression in Bone Defects

Endogenous bone morphogenetic protein expression in fracture sites has been shown to be crucial for the initiation and progression of fracture healing *in vivo* [97]. Conversely, the overexpression of the native BMP antagonists Noggin and Gremlin has been shown to impair bone formation and fracture healing both *in vivo* and *in vitro* by reducing BMP activity [98–100]. Overexpression of Noggin in mice impairs osteoblastic differentiation and expression of markers of osteogenic differentiation and results in a net loss of bone density, decrease in rate of bone formation, and increase in fractures without an increase in osteoclast formation [98, 100]. Similar studies of overexpression of Gremlin demonstrated reduced bone volumes, a decreased response of bone-forming cells to BMPs- and an increase in the rate of fractures [99].

Additional studies of limb fracture healing in a limb-specific BMP-2-deficient mouse model suggest that even in the presence of additional osteogenic factors, BMP-2 is necessary for the initiation of fracture healing [101]. In the complete absence of BMP-2 expression in nullizygous mouse limbs, bones are completely incapable of initiating a regenerative response to fracture, and a dose-dependent decrease in bone density is seen in BMP-2 heterozygotes [101].

Studies of BMP expression in fractures of the craniofacial skeleton also confirm involvement of BMPs in the native craniofacial fracture healing process. BMP activity, along with the activity of other members of the TGF-β superfamily and postreceptor signaling through Smad proteins, has been shown to be elevated in native craniofacial fracture healing by intramembranous ossification during distraction osteogenesis of the mandible in rats and sheep following osteotomy [102–104].

Along with other previous well-described findings concerning the osteogenic properties of BMPs, findings such as these have suggested that the additional exogenous application of BMPs may augment the natural role of intrinsically secreted BMPs in producing osteogenesis in the setting of fracture or reconstruction of the craniofacial skeleton. Numerous animal studies have explored this possibility [102–104].

5. Roles of the Bone Morphogenetic Proteins in Osteogenic Differentiation

Most studies of osteogenesis using bone morphogenetic proteins have focused on BMP-2 and BMP-7, likely as a result of the commercial availability of recombinant human forms (rhBMP-2 and rhBMP-7) and their clinical applications in nonunion fracture healing and spinal fusion [40, 88]. Though commonly used in research and clinical applications, BMP-2 and BMP-7 had not been previously proven to be the most potent inducers of osteogenesis in the BMP family prior to their adoption in clinical and research settings [105]. The creation of recombinant adenoviruses for the 14 different BMPs (AdBMPs), many of which had previously been unavailable for research use due to a lack of a recombinant or bioactive recombinant form, has resulted in a better understanding of the osteogenic potential of the different subtypes [43, 105].

In vitro and *in vivo* studies of AdBMP-transfected mesenchymal stem cells confirmed the osteogenic potential of BMP-2, 4, 6, 7, and 9 [43]. Studies of BMP-3 in knock-out mice had previously demonstrated that the knock-out condition resulted in an increase in bone density, demonstrating its role as a negative regulator of osteogenesis [106]. Current evidence suggests that BMPs 4 and 7 are the weakest of the osteogenic inducers of ossification in the BMP family and BMPs 2, 6, and 9 are the strongest [41, 43]. BMP-9, one of the least studied members of the BMP family, is the most potent overall and is the strongest inducer of osteogenesis both *in vitro* and *in vivo* [40, 42, 88, 107].

Interestingly, BMP-9-induced osteogenic differentiation, unlike the osteogenic differentiation induced by the other osteogenic BMPs (2, 4, 6, and 7), has been shown to be unimpaired by the presence of BMP-3 [37, 40, 43, 89]. This finding may indicate that in addition to being the most potent inducer of bone formation, BMP-9 may also induce osteogenesis and osteogenic differentiation of mesenchymal stem cells through a signaling pathway, that is, overlapping, but also distinct from the signaling pathways shared by the other osteoinductive BMPs. Additional evidence supports this theory, as BMP-9 has been shown to have a relatively lower affinity for the BMPR-IA receptor utilized by other osteogenic BMPs [108, 109]. Further evidence which could explain the unique characteristics of BMP-9 is the presence

of a retained N-terminal region not found in other secreted BMPs or other members of the TGF-β superfamily; this feature has been postulated to provide increased stability to BMP-9 following secretion [87, 109]. While BMP-9 has great promise for use in clinical and experimental scenarios, its safety for clinical use has not yet been established. Further studies not only into the safety of BMP-9 but also the mechanism and normal function of BMP-9 in osteogenesis are warranted.

6. The Use of BMPs in Experimental Models of Craniofacial Defects

Numerous studies of critical-size calvarial, alveolar, and mandibular defect models of BMP-induced osteogenesis have demonstrated a successful result in a wide variety of animal models. These have focused on the delivery of BMPs, typically rhBMP-2 and rhBMP-7, with a wide variety of delivery systems including demineralized bone matrix and other alloplastic materials along with the standard commercially available collagen sponge implants [15, 94, 110–120].

A recent study by Hassanein et al. describes the comparative analysis of rhBMP-2, split thickness calvarial bone grafts, and calvarial particulate bone grafts for the repair of calvarial defects in rabbits [110]. The study authors used these grafts to repair 17×17 mm parietal bone defects and found no significant differences in defect coverage between experimental groups; however, rhBMP-2-repaired defects produced thinner bone than both split thickness calvarial and calvarial particulate bone grafts [110]. There was no difference in bone regenerate thickness between split thickness and particulate bone grafts [110].

Skull bone regeneration of critical-size defects using rhBMP-2 has also been achieved in nonhuman primates. In a study by Takahashi et al., biodegradable hydrogels were used to deliver rhBMP-2 to a defect site created in cynomolgus monkeys and were found to be capable of inducing repair even at low doses. As the native bone bridges the gap, the hydrogel is degraded and releases BMP. The bone regenerators were not compared to autologous bone grafts but were compared to insoluble bone matrix containing rhBMP-2, and the biodegradable hydrogels were found to induce greater bone regeneration [121]. These findings suggest that the use of a delivery system that mimics the natural release of BMP in native fracture healing for exogenous administration of BMPs may yield enhanced bone regeneration.

Of particular note for pediatric craniofacial reconstruction in the calvarium, rhBMP-7 implantation has been evaluated in a growing bone model of critical-size calvarial defects in infant mini-pigs [118]. Four months after the experimental, period both the control group (autologous bone graft) and the experimental group (rhBMP-7) demonstrated equivalent histologic quality of bone regeneration and the regenerated bone maintained the structure, density, and growth expected of the native calvarium in the area of the defect. These findings suggest that BMPs may provide a good solution for craniofacial defects in the growing pediatric skeleton.

A notable study also examines the ability of BMPs to regenerate bone in the unfavorable setting of irradiated tissue. Patients requiring mandibular resection for head and neck cancer often face the additional challenge to reconstruction posed by healing and tissue regeneration in the setting of a therapeutically irradiated site. The expression levels of BMP-2 and BMP-4 have been shown to be decreased in experimentally irradiated bone [122]. Subsequent experimental application of BMP-2 to the mandibles of rats in an irradiated bone model has been shown to provide regeneration equivalent to that of nonirradiated tissue following mandibular resection [123]. These results suggest that a significant obstacle to bone tissue regeneration in an irradiated site may be reduced levels of local BMP expression and secretion and suggest a possible role for BMP therapy in patients following radiation therapy for head and neck cancers.

7. BMP-Induced Inflammation in Experimental Models

In addition to human clinical reports of BMP implant-associated edema, BMP-induced inflammation has also been observed in rodent models of soft tissue inflammation in response to exogenous BMP application [46, 47]. MRI measurements of tissue edema volumes following administration of high doses of rhBMP-7 and rhBMP-2 showed that rhBMP-7 produced an inflammatory response than rhBMP-2 implants in the same setting [46, 47]. The tissue inflammation was also shown to be reduced when the rhBMPs were delivered in the presence of bone morphogenetic protein binding peptide, which functions as a slow release carrier for the BMPs in experimental models [46]. These findings suggest that under the appropriate conditions for BMP delivery, the adverse inflammatory responses may be abated.

8. Clinical Use of BMPs in the Human Craniofacial Skeleton

Some of the earliest reports of the clinical use of BMPs partially purified from bovine bone describe successful application of these BMPs along with titanium implants or lyophilized cartilage in the reconstruction of various craniofacial deformities found the in Apert and Crouzon syndrome [124, 125]. Commercially available rhBMP-2 is only FDA approved for intraoral applications in the craniofacial skeleton; however, off-label use of rhBMP-2 and rhBMP-7 has been attempted in some cases to solve other particularly significant reconstructive challenges in the craniomaxillofacial skeleton [29, 48, 126, 127]. Notable cases in the craniomaxillofacial skeleton involve the reconstruction of the cranial vault, the reconstruction of alveolar cleft deformities, and the reconstruction of mandibular defects.

9. rhBMP-2 for Human Cranial Vault Reconstruction

The first and only reported off-label use of rhBMP-2 for cranial vault reconstruction in the medical literature is a case report from the Washington University in Saint Louis that describes reconstructions of the cranial vault using

rhBMP-2 in pediatric patients and a subsequent notable case of adverse scalp and facial edema that required surgical discontinuation of the graft [48]. The authors describe the occasional off-label clinical use of rhBMP-2-infused collagen sponge implants in children older than 18 months of age with significant cranial defects created during cranial reconstruction procedures that would otherwise need to be repaired by autologous bone tissue grafting. Postoperative scalp, periorbital, and facial edema following surgical craniosynostosis correction usually resolves at the conclusion of the first postoperative week [48]. In the case described, a patient undergoing craniofacial reconstruction for metopic craniosynostosis experienced marked scalp, periorbital, and facial edema extending to the anterior cervical region without compromise of the patient's airway. A one-week glucocorticoid taper was used to successfully reduce the swelling until the patient's discharge; however, upon discontinuation of the steroid course on post-operative day 7, the patient redeveloped scalp and facial edema. The patient's collagen sponge rhBMP-2 implants were removed surgically on postoperative day 10 and the swelling rapidly remitted and did not return. These results corroborate findings of adverse events related to tissue swelling and edema in previously documented case reports of rhBMP-2 use in the cervical spine, where the regional anatomical risk of adverse patient outcomes related to potentially fatal airway edema is significantly greater than that in the craniofacial region [49, 50].

10. rhBMPs for the Reconstruction of Human Clefts

A number of clinical studies and randomized controlled trials have demonstrated effective repair of congenital human alveolar clefts by the use of exogenously applied rhBMP, and a review of the literature is available on this subject [20–25, 128] (Table 1).

The earliest reported cases of rhBMP application to bony cleft repair from Carstens et al. and Chin et al. at Saint Louis University document successful repair of congenital facial clefts with rhBMP-2. In the Chin et al. case series, 50 clefts were repaired with rhBMP-2 in 43 patients with successful clinically assessed reconstruction achieved in 49 of 50 clefts; however, radiographic evidence of repair was not provided in this study and several severe cases were additionally treated with distraction osteogenesis [20]. The Carstens et al. study describes a more complicated case where a complete cleft and mandibular defect were repaired by application of distraction assisted in situ osteogenesis (DISO) and rhBMP-2; a collagen sponge saturated with rhBMP-2 was implanted following DISO to generate new bone which was used to repair the cleft and form new bone to graft onto the deformed mandible [21]. Later, a 2009 retrospective cohort study, also from Saint Louis University, assessed the off-label use of rhBMP-2 in seventeen clefts six months postoperatively using spiral CT [24]. Sixteen of seventeen cleft repairs were found to have both vertical and transverse filling of the maxillary cleft by new bone at six months by spiral CT evaluation [24].

In 2007 Herford et al. reported a retrospective review of twelve patients undergoing cleft repair, with ten patients receiving rhBMP-2 collagen sponges and two patients receiving autologous iliac bone grafts [22]. Radiographic evaluation at four months was performed via computed tomography (CT) and showed significant development of bone in eight out of ten patients in the experimental group with two of those eight patients having significantly less bone formation than the group average [22]. On average, bone volume generation was found to be slightly less (7%) in the rhBMP-2 treatment group than in the ABG group. Significant postoperative swelling was noted by the authors in the rhBMP-2 experimental group [22].

A 2009 study by Dickinson et al. describes the randomization of skeletally mature unilateral cleft patients with alveolar defects to experimental groups using either autologous iliac bone grafting or an rhBMP-2 prepared resorbable collagen matrix following preoperative orthodontic maxillary expansion. CT evaluation of bone formation in both groups at 12 months showed that the rhBMP-2 group had greater volume of the defect filled (93%) when compared to the autologous iliac bone graft group (63%) [23]. The ABG control group also had significantly greater length of stay, cost, more wound healing problems, and increased pain as a result of the donor site graft harvest [23]. These findings are unique to this study and suggest that skeletally mature patients undergoing cleft repair may have better outcomes with the use of rhBMP-2 than autologous bone grafting; these findings warrant further investigation due to the small sample size of this study.

In 2011 Alonso et al. reported the use of resorbable collagen matrix with rhBMP-2 or iliac crest ABG in a randomized controlled trial for the repair of unilateral cleft lip and palate in skeletally immature 8–12-year-old patients following preoperative orthodontic maxillary expansion [25]. Average bone volume measured by CT was found to be slightly less (5.8%) in the rhBMP-2 treatment group when compared to the ABG group, but good outcomes were achieved in both experimental groups [25]. Significant postoperative swelling was reported in the experimental group only with 37.5% of patients experiencing this effect [25].

11. rhBMPs for Human Mandibular Reconstruction

Several clinical cases describing the use of rhBMPs for the reconstruction of human mandibular defects have been reported in the past decade [4, 26, 29, 44, 126, 129–131] (Table 2).

The first reported human clinical application of bone morphogenetic protein to reconstruction of the mandible was the successful reconstruction of a 6 cm mandibular defect by application of a poloxamer-based gel containing bone morphogenetic proteins isolated and partially purified from allogenic bone samples [26]. Radiographic confirmation of bone formation was performed at three and nine months, and a bone biopsy at nine months provided histological confirmation of the formation of bone containing healthy osteocytes.

In 2002, clinicians at the same institution performed a randomized controlled trial for the reconstruction of

Table 1: Selected clinical studies using rhBMPs for human cleft reconstruction.

Authors	Year	Description	Main findings
Chin et al. [20]	2005	Case series using rhBMP-2 in 50 clefts, 43 patients	Successful clinical reconstruction in 49/50 clefts; no radiographic evidence provided; several cases treated with distraction osteogenesis
Carstens et al. [21]	2005	Case report using rhBMP-2 in one patient	Distraction assisted in situ osteogenesis followed by rhBMP-2 collagen sponge implantation; new bone in cleft site used to graft onto and reconstruct deformed hemimandible
Herford et al. [22]	2007	Retrospective review using rhBMP-2 ($n = 12$)	Significant filling of maxillary cleft by new bone in eight out of ten patients in the rhBMP-2 experimental group at four months on CT
Dickinson et al. [23]	2008	Randomized controlled trial using rhBMP-2 ($n = 21$)	Cleft repairs of skeletally mature patients found to have greater volume of defect filled by new bone measured by CT in the rhBMP-2 group (93%) when compared to the ABG group (63%), a unique finding
Fallucco and Carstens [24]	2009	Retrospective cohort study using rhBMP-2 ($n = 17$)	Vertical and transverse filling of maxillary cleft by new bone seen with spiral CT at six months in sixteen of seventeen cleft repairs
Alonso et al. [25]	2010	Randomized controlled trial using rhBMP-2 ($n = 16$)	Average bone volume measured by CT 5.8% less in rhBMP-2-treated group than in ABG-treated group

ABG: autologous bone graft; CT: computed tomography; rhBMP: recombinant human bone morphogenetic protein.

segmental mandibular bone defects in thirteen patients using a titanium mesh supplemented with either autologous bone grafts (ABGs) from the iliac crest or an osteogenic device composed of demineralized bone matrix reconstituted with bone morphogenetic proteins harvested and partially purified from allogenic bone [27]. Upon biopsy at three months, two out of the six patients treated with the osteogenic device had histologic evidence of bone formation and five out of the seven patients treated with ABGs had histologic evidence of bone formation.

A unique 2004 case report from Germany documents the use of an exogenously prepared customized mandibular bone graft grown in the latissimus dorsi muscle of the patient prior to its use for reconstruction of a large mandibular defect [28]. The graft was prepared by filling a customized titanium mesh outer scaffolding with an amalgam of hydroxyapatite blocks coated with rhBMP-7 and autologous bone marrow mesenchymal stem cells. This preparation was subsequently implanted into the patient's latissimus dorsi muscle for seven weeks to allow for vascularization, ossification, and bone remodeling within the graft prior to reconstruction of the mandible. Implantation of the graft into the latissimus dorsi effectively allowed the muscle to serve as a human bioreactor prior to subsequent free flap transfer into the mandibular defect. The patient's postoperative course was complicated by fracture of the scaffolding near the docking points of the mesh to the proximal mandible stumps. The fracture caused exposure of the implant to oral flora resulting in infection of the implant and necrosis of areas of bone inside the mandible. The necrotic areas were removed surgically and the remaining healthy bone portion of the implant remained in place in the patient until his death from a cardiac arrest fifteen months later. Radiation therapy at the location of the implant, the patient's refusal to stop smoking, and consumption of hard nut candy and heavy meals may have influenced the development of morbidities.

Two case series document the use of rhBMPs in mandibular reconstruction, one using collagen sponges containing rhBMP-2 and another using demineralized bone matrix reconstituted with rhBMP-7 [4, 29].

One of the above studies utilizing rhBMP-2 was able to demonstrate successful restoration of mandibular continuity in three out of five patients receiving rhBMP-2 implants. Investigators in this study report that four out of five patients experienced significant facial swelling far greater than would be expected in the case of autologous bone graft reconstruction [29]. Successful restoration of mandibular continuity using rhBMP-2 has also been reported by Herford et al. in case reports featuring three different patients [45, 127, 129, 131, 132].

In the study by Clokie and Sándor, successful restoration of mandibular continuity was achieved in all ten patients using the rhBMP-7 reconstituted demineralized bone matrix and rigid reconstruction plates. Swelling and induration at the site of implantation were reported in all ten cases but resolved 4 weeks postoperatively [4].

Overall, these clinical cases provide strong evidence that rhBMPs are capable of inducing osteogenesis in the setting of mandibular defects, though they are not as reliable or effective as the gold standard of autologous bone grafting. A review article summarizing these clinical studies reports an overall 13.5% failure rate of rhBMP mandibular reconstructions excluding the exogenously prepared implant described by Herford et al. [45].

Table 2: Selected clinical studies using rhBMPs for human mandibular reconstruction.

Authors	Year	Description	Main findings
Moghadam et al. [26]	2001	Case report using poloxamer-based gel containing BMP partially purified from allogenic bone	Successful clinical result; radiographic confirmation of bone formation at 3 and 9 months; biopsy for histological confirmation of bone formation at 9 months
Ferretti and Ripamonti [27]	2002	Randomized controlled trial using demineralized bone matrix reconstituted with BMP partially purified from allogenic bone ($n = 13$)	Biopsy at three months showed bone formation in two out of six patients treated with BMP and five out of seven treated with ABG
Warnke et al. [28]	2006	Case report of reconstruction with exogenously prepared titanium mesh graft filled with rhBMP-7-coated hydroxyapatite blocks	Successful clinical functional result achieved following free flap transfer of the customized mandibular graft to the maxilla following 7-week incubation in the latissimus dorsi
Clokie and Sándor [4]	2008	Case series using rhBMP-7 reconstituted demineralized bone matrix in ten patients	Successful radiographic and functional restoration of mandibular continuity in all 10 patients
Carter et al. [29]	2008	Case series using rhBMP-2-soaked collagen sponges alone or in combination with bone marrow cells and allogenic cancellous bone chips; five patients, four with mandibular continuity defects and one with two large bone cavities in the mandible	Restoration of mandibular defects in 3 out of 5 patients assessed clinically and by radiograph; two patients with failed reconstruction had mandibular continuity defects

ABG: autologous bone graft; CT: computed tomography; rhBMP: recombinant human bone morphogenetic protein.

12. Isolation, Purification, and Culture of Adult Mesenchymal Stem Cells

Techniques for the identification, isolation, purification, and subculture of human bone-marrow-derived mesenchymal stem cells (BM-MSCs) have been previously established in many studies [133, 134, 134–136]. Mesenchymal stem cells derived from human bone marrow can be cultivated, cryopreserved, maintained in a progenitor state, and expanded over one-billion-fold *ex vivo* [133, 137]. Bone marrow mesenchymal stem cells are multipotent and capable of subsequently being driven down differentiation pathways to bone, cartilage, adipose, and connective tissue [133, 138–140]. In addition to BM-MSCs, adult adipose-derived stem cells have also been shown to have osteogenic potential and have been successfully used with synthetic scaffolds to repair critical-size calvarial defects in a mouse model [141, 141–147]. Clinically, the use of osteoblast precursors may be limited by the lifespan of these cells both in culture and following implantation into patients. Strategies for immortalization of these cells, such as adenoviral gene therapy with human telomerase reverse transcriptase, have been developed and successfully used to bridge critical-size defects in animal models; however, the potential for tumorigenesis of immortalized cell populations poses a significant risk to patients [147, 148]. Though protocols have been described for isolation of these cells via collection from marrow and isolation of MSCs by adherence to culture plates in various media, the process is not standardized and may not currently be optimized [135, 138, 139, 149]. Despite the lack of a definitive protocol for isolation and expansion of human populations of osteoblast precursors, these multipotent stem cells provide a fertile ground for the application of bone morphogenetic proteins for use in developing bone-forming tissue *ex vivo*.

13. Paradigm for Osteogenic Differentiation of MSCs

The tissue engineering strategy of *ex vivo* isolation, expansion, and differentiation of adult-derived mesenchymal stem cells by exposure to BMPs is a promising alternative to autografts, allografts, and alloplastic bone substrate materials in craniofacial repair. The transfer of these personalized and modified osteoprogenitor cells to a critical-size osseous defect has high therapeutic potential for repair of craniofacial deformities caused by trauma, tumor, infection, or congenital disease in both adult and pediatric patients. A significant advantage in the use of mesenchymal stem cells derived from a patient's own tissues is that these modified cells can be used without the threat of many of the potential sources of complication and morbidity associated with allografts and alloplastic materials, for example, the morbidity associated with insufficient biocompatibility of alloplastic materials and the sterility of alloplastic materials and allografts can be largely avoided with the use of mesenchymal-stem-cell-derived osteoblasts. Personalized *ex vivo* differentiated osteoblasts, unlike alloplastic materials, can also fulfill the need for a stable biological substrate that can grow and adapt over time, a crucial feature for reconstruction in both adults and children.

Furthermore, implantation of osteoblastic cells differentiated *ex vivo* may provide a substrate superior to direct implantation of BMPs or BMPs delivered by adenoviruses. Exogenous applications of BMPs can cause many clinical morbidities including but not limited to the persistent

formation of ectopic bone nodules, highly accelerated, unregulated bone growth that outstrips the ability of surrounding tissues to provide vascular and nutritive support and localized inflammatory reactions [32, 33, 48, 128]. Additionally, defects and loss of control in the differentiation of mesenchymal stem cells have been shown to be responsible for the formation of human bone and soft tissue cancers such as osteosarcoma [150, 151]. Unfortunately, the populations of cells exposed to exogenous BMPs cannot be monitored *in vivo* for the development of neoplasia. The danger of tumorigenesis is present with the application of recombinant protein and virus; however, it is especially apparent in the setting of gene therapy where the dose of BMP delivered is not tunable (i.e., there is no "off" switch). Thus, a significant obstacle to therapeutic application of exogenously delivered BMPs is the lack of control of tumorigenesis of the exposed cell population. In light of these dangers, exogenous BMP therapy should be approached with significant caution.

The adverse outcomes associated with direct exogenous or adenoviral delivery of BMPs to bony defects could be prevented by the application of personalized bone-forming cells. A significant advantage of *ex vivo* tissue engineering strategies is the control of tumorigenesis of the expanded cell population; expanded populations of osteoblastic cells differentiated from MSCs could be tested to ensure that cancerous cells are not transferred back to the patient. Strategies incorporating MSCs for use in craniofacial repair are not exempt from difficulty, however, as current techniques for isolation and expansion of cells are expensive and time consuming. In addition, the application of *ex vivo* engineered cell populations and bone tissue may be limited by the potentially limited life span of mesenchymal stem cells and osteoblastic cells *in vivo*. Further research into the improvement of these techniques may make the use of personalized stem cells an efficient and feasible approach for patients facing craniofacial reconstruction in the future.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Toward Personalized Cell Therapies by Using Stem Cells: Seven Relevant Topics for Safety and Success in Stem Cell Therapy

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Stem cells, both embryonic and adult, due to the potential for application in tissue regeneration have been the target of interest to the world scientific community. In fact, stem cells can be considered revolutionary in the field of medicine, especially in the treatment of a wide range of human diseases. However, caution is needed in the clinical application of such cells and this is an issue that demands more studies. This paper will discuss some controversial issues of importance for achieving cell therapy safety and success. Particularly, the following aspects of stem cell biology will be presented: methods for stem cells culture, teratogenic or tumorigenic potential, cellular dose, proliferation, senescence, karyotyping, and immunosuppressive activity.

1. Introduction

The study of the stem cells potential has stimulated the onset of new areas, as the regenerative medicine and tissue bioengineering. Cell-based therapies to treat human diseases have become a clinical reality in the light of the advances in research with adult stem cells and embryonic stem cells, the two major divisions of stem cells. Recent works have shown that it is already possible to reprogram somatic cells into ones with similar characteristics to ESCs, being referred to as induced pluripotent stem cells (iPSCs). These cells are strong candidates to be applied in cellular therapy, requiring even more studies to master this new technology.

PubMed searches for publications with the exact terminology "stem cell therapy" indicate a strong growth in the number of publications in this area over the last 19 years, shown in Figure 1. In spite of the obvious importance of this issue, only few manuscripts appeared before 2000 years.

Aiming for a personalized cell therapy, some criteria or parameters must be observed. This paper will discuss some controversial issues of importance for achieving cell therapy safety and success. Particularly, the following aspects of stem cell biology will be presented: methods for stem cells culture, teratogenic or tumorigenic potential, cellular dose, proliferation, senescence, karyotyping, and immunosuppressive activity.

2. Cell Culture Free of Animal Components

Methods of cell cultivation have been fundamental to physiological, biological, and pharmacological assessments at cellular and tissue levels [1], as well as to molecular studies. Besides enabling the production of biological components of human interest, such as vaccines and hormones, cell cultures help in the advancement of stem cell research by

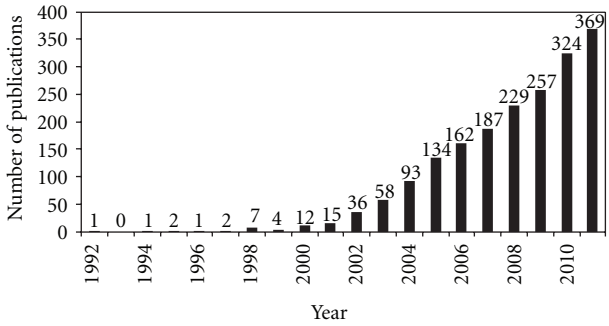

Figure 1: Published articles from 1992 to 2011. The information was retrieved from PubMed indexed articles using the exact terminology "stem cell therapy."

allowing the improvement of the stem cells for cellular therapy. The cultivation conventional system of mammalian cells is performed in an incubator at 37°C (5% CO_2 and 95% O_2). The composition of the culture medium is critical to the success of cell culture and the maintenance of cells for long periods *in vitro*, often requiring to be supplemented with other factors, such as serum. The most commonly used serum is fetal bovine serum (SFB) which is composed of a mixture of several substances in undefined concentrations as hormones, growth factors, vitamins, and other unknown substances [1].

The use of serum in culture media in research is associated with several problems such as limited availability of good fetal bovine serum suppliers, batch-to-batch variation causing inconsistency in both cell growth pattern and products formation and the risk of viral, mycoplasmal, and prions contamination, and interfering with the effect of hormones or growth factors upon studying their interaction with cells [2–4]. Due to the possible contamination of stem cells with bovine infectious agents and the concern of transmission of these agents to patients, plus the possibility of cultured cells to incorporate animal proteins that can provoke allergic reactions in humans [4], there is a growing concern and a consensus that research has to be done in order to establish new supplements and cultivation conditions for cell therapy, avoiding the use of animal-origin reagents in cell culture.

There are more than 100 serum-free culture medium formulations [5] with 4 basic types of culture medium [1]: (i) serum-free medium; containing small fractions of protein either from animal origin or from plant extracts which characterize it as a medium chemically undefined; (ii) protein-free medium; it has fractions of peptides (hydrolysed proteins) and is also considered to be a chemically undefined medium; (iii) animal- and human-derived components-free medium; it is not considered to be chemically defined, since it may contain bacteria, yeast hydrolysates, and plant extracts; (iv) chemically defined medium; it does not have protein, hydrolysed or any component of unknown composition. Hormones, animal or plant growth factors, as well as highly purified recombinant products may be supplemented in the culture medium.

Many research groups have been struggling to achieve the appropriate culture medium for cell therapy; however, many searches need to be performed to achieve this purpose.

3. Teratogenic or Tumorigenic Propriety of Stem Cells

The stem cells can be classified as embryonic (ESCs) and adult stem cells (ASCs). ESCs research reveals their enormous potential for differentiation and ability to originate almost all tissues of our body. The establishment of ESCs lines is problematic, since it involves ethical issues in relation to the destruction of embryos. According to the literature, there are several reports of ESCs transplants performed on animals, which resulted in the formation of teratomas on the recipient organism [6–14], which is undesirable for cellular therapy. Particularly, teratomas have been formed by hESC transplantation into the testis [15–17], kidney capsule [18], liver [19], hind leg muscle [20–23], and into the subcutaneous space [19, 24].

Numerous published reports have examined the potential of differentiated cell types derived from mouse and human ESCs to repair nonhuman target organs in intact animals [25–33]. Recent studies, however, have yielded both encouragement and caution, with restoration of function evident to some degree in many cases, but coexisting in others with the troubling finding that the grafts contained evidence of cancerous growth [8, 14, 34–38].

Kahan et al. [39] showed that the selection of embryonic stem cells presenting the markup $SSEA1^{-}SSEA3^{-}EpCAM^{+}$, both mouse and human, resulted in eliminating the tumorigenic potential from differentiated ESC populations. Sorted cells do not form teratomas after transplantation into immunodeficient mice, but showed gene and protein expression profiles that are indicative of definitive endoderm cells. Sorted cells could be subsequently expanded *in vitro* and further differentiated to express key pancreas specification proteins. *In vivo* transplantation of sorted cells resulted in small, benign tissues that uniformly express PDX1. It represents one of transcripts expressed during gastrulation or early periods of endoderm development.

Adult stem cells have generated great interest among the scientific community devoid of their potential therapeutic applications for unmet medical needs. According to some studies, in addition to ESCs, some adult stem cells also form tumors when reintroduced into the organism. Recently, it was reported a case of a child with ataxia telangiectasia that developed multifocal brain and spinal cord tumors 4 years after treatment with human neural stem cells originating from at least two donors, even though the cells were relatively freshly derived from chromosomally normal fetuses [40]. Among ASCs, we highlight the hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) as those most well studied and reported in the scientific literature.

Obtaining the HSCs from bone marrow is an invasive and painful process for the patient. The HSCs are more restricted, as they have the capacity of proliferation and differentiation, resulting only in cells of the myeloid and

lymphoid lineages. The transplant with HSCs must also be careful because it can result in graft-versus-host disease (GVHD) [41, 42]. This is a systemic syndrome that occurs in patients that receive immunocompetent lymphocytes. The pathophysiology involves an immune reaction between transplanted lymphocytes and development of an immune attack of the T cells from the donor to the host's cell, which differ from the former by histocompatibility antigens. It is, therefore, a primary complication of allogeneic bone marrow transplantation [41, 42]. The first three months after transplantation of HSCs can be marked by a greater number of complications due to direct toxicity of the conditioning, coupled with prolonged period of aplasia, infections, and acute GVHD. After this period and in the months ahead, complications are less frequent but also occur, as a result of chronic GVHD, with late damages to several organs and hematopoietic system [43]. For these reasons, HSCs should also be used with caution. In accordance with the literature, the HSCs obtained by ESCs differentiation can result in teratomas when reintroduced into the body. Its use in cell therapy should ensure that the transplanted cells will be free of residual teratogenic cells [44–47].

Besides the HSCs, there is another cell type present in the bone marrow which has great potential for cell therapy, the mesenchymal stem cells (MSCs). MSCs have been identified from various tissues in the past decade, including bone marrow, adipose tissue, umbilical cord, and dental pulp. The International Society for Cellular Therapy has recommended the following minimum criteria for defining multipotent human MSCs [48, 49]: (i) adherence to plastic under standard culture conditions; (ii) positive for expression of CD105, CD73, and CD90 and negative for expression of the hematopoietic cell surface markers CD34, CD45, CD11a, CD19 or CD79a, CD14 or CD11b, and histocompatibility locus antigen (HLA)-DR; (iii) under a specific stimulus, differentiation into osteocytes, adipocytes, and chondrocytes *in vitro*. To date, human bone marrow represents the major source of MSCs [50–54], and MSCs have potential to be expanded and cryopreserved for future use as an off-the-shelf therapy [55]. Currently, autologous MSCs derived from bone marrow have been applied for cell-based therapies, including the treatment of osteogenesis imperfecta, intracoronary transplantation in patients with acute myocardial infarction, and support of haematopoiesis [50, 51, 53, 54, 56]. Studies in mice show that MSCs are also involved in carcinogenesis. Houghton et al. [57] showed that chronic infection of C57BL/6 mice with *Helicobacter*, a known carcinogen, repopulated the stomach with bone-marrow-derived cells. It was observed that these cells progressed through metaplasia and dysplasia to intraepithelial cancer. These findings have broad implications for the multistep model of cancer progression, as they suggest that epithelial cancers can be originated from bone-marrow-derived sources [57]. Thus, a study of carcinogenic potential sources of many adults MSCs is prudent.

The reprogramming of somatic cells was a major advance in order to avoid ethical problems related to the destruction of the embryo, and this methodology has helped understand even more about pluripotency. There is an effort to improve the technique, since many use retroviral or lentiviral vectors for expression of genes related to embryonic transcription factors (NANOG, OCT4, and KFL4) besides c-Myc. The reprogrammed cells exhibit characteristics similar to embryonic stem cells, the ability to differentiate into many cell types, but there are also several reports of teratoma formation [58–60].

4. Appropriate Cellular Dose to Be Transplanted

One of the major issues to be addressed is the cell dose to be transplanted into a patient, which would provide successful treatment. As well as medicines, there must be a correlation between cell number and the body weight of the patient. According to the literature, there is no consensus among researchers about the cell dose required.

Currently, the most used cells in cell therapy are hematopoietic stem cells present in umbilical cord blood (CB) and bone marrow and, in some cases, in the peripheral blood. Human umbilical cord blood has long been recognized as a rich source of primitive and committed hematopoietic progenitors. In addition, the general availability and the ease of procurement make cord blood a very attractive alternative source of transplantable hematopoietic tissue [61]. HSCs are contained within a population of mononuclear CD34+ antigen-expressing cells, which typically represent less than 1% of the total leukocytes in CB [62]. Research have suggested that, on the basis of the number of progenitor cells present in umbilical cord blood, it needs to be transplanted with restriction on children and some adults weighing less than 40 kg [63–65].

Jaime-Pérez et al. [66] analyzed 794 CB units in which the amount of $CD34^+$ cells was determined by flow cytometry. Although there are not accepted universal guidelines, most Cord Blood Banks use the combination of product weight (volume) and total nucleated cell (TNC) count as the main selection factors for cryopreservation, requiring a TNC content from $6–10 \times 10^8$ for storage [67] and a minimal volume between 40 and 60 mL [68–70]. The study showed that the amount of TNC is the best parameter that correlates with the contents of CD34+ cells, being in agreement with previous reports [71–73] and that all CB units having a TNC count of 8×10^8 or more had the required CD34+ cell dose for patients weighing 10 kg or less.

Even in the face of such an impasse, the use of cord blood has been extended to include adults, allowing better definitions of cell dose limitations and thresholds [74–79]. The results of these transplants have helped to define a requirement for a minimum cell dose from 3×10^7 to 3.5×10^7 nucleated cells/kg in order to obtain acceptable clinical outcomes [80], and specifically, the CD34+ cell content has been shown to influence engraftment and survival after unrelated UCB transplantation, better predicting the hematopoietic potential of a CB unit was obtained after infused with 1.7×10^5 CD34+ cells per kilogram of the recipient's body weight the threshold dose than nucleated cell content [81].

Nucleated cells can also be obtained from the bone marrow and peripheral blood. Hernigou et al. [82], aiming

for autologous transplantation, calculated the number of medullary nuclear cells per kg of marrow using a formula that takes into account blood dilution. In each milliliter of aspirate, it was estimated that medullary cells were represented by the difference between the nuclear cell count in the aspirate and that in peripheral blood, which is assessed during general anesthesia. The number of nuclear cells of presumed medullary origin per kg is expressed as follows: $N(10^8/\text{kg}) = (V \times \text{NP}) - (V - 100) \times \text{NS/P}$, where V is total volume of aspirate in mL, including the harvesting medium; NP is nuclear cell count per milliliter in the collection bag which leaves the operating room, including the harvesting medium; $V - 100$ is the exact volume of aspirate, after subtraction of the 100 mL of harvesting medium; NS is the nuclear cell count per milliliter of peripheral blood drawn during general anesthesia; P is patient's weight in kilograms. Thus, for these parameters researchers suggest a total final volume of 300 mL containing 14×10^6 nuclear cells per milliliter, obtained from a 70 kg adult with a leucocyte count of 4×10^6 per milliliter as determined under general anesthesia, it may be estimated that the medullary nuclear cell count is 5×10^7 per kilograms, for a total of 0.35×10^{10} nuclear cells [82]. In case of allogeneic transplants, it is recommended to be obtained a number of nucleated cells greater than 2×10^8/Kg. This way, when the weight of the donor is similar to the weight of the receptor, this value is obtainable by the aspiration of nearly 10 mL/kg of the weight of the donor, by volume of aspired bone marrow [83].

In peripheral blood, under normal conditions, there is a small number of stem cells (CD34+). Therefore, the use of this route to obtain stem cells for medullar transplant requires the prior mobilization of these cells from the bone marrow to the blood [84]. This mobilization is done by the administration of recombinant hematopoietic growth factor or colony-stimulating factor (CSF) to obtain a sufficient concentration of blood $CD34^+$ cells in the blood and ensure the success of the transplantation [84, 85]. The optimal number of CD34+ cells for allogeneic transplantation is not well established and it is usually done with 4 to 6×10^6 CD34+ cells/kg of the receiver's weight [86–89]. In autologous transplant, the recommendation is that the number of cells must be greater than 2×10^6 CD34+ cells/kg of the patient's weight [90].

5. Proliferation, Senescence, and Karyotype

The embryonic and adult stem cells may undergo symmetric cell divisions to self-renew or undergo terminal differentiation, or they may undergo asymmetric cell divisions to generate differentiated progeny as well as maintain a pool of stem cells. A dynamic balance between proliferation, survival, and differentiation signals ensures that an appropriate equilibrium between stem cells, precursor cells, and differentiated cells is maintained throughout development and adult life [91].

This renewal capacity is not a perfect process and the "daughter's cells" gradually lose the ability to proliferate, partly due to a gradual erosion of telomeres in each cell division. This phenomenon can be observed both *in vivo* and *in vitro* [92–95]. Telomeres are structures present at the end of eukaryotic chromosomes that protect chromosomes from degradation, fusion, and recombination. In mammalian cells, they consist of hexanucleotide (TTAGGG) repeats and several associated protein components. In the absence of compensatory mechanisms, dividing cells undergo gradual telomere reduction. When telomeres reach a critical degree of shortening, cells recognize this as DNA damage and initiate proapoptotic programs or enter senescence [96].

In vivo, as the body ages, the stock of stem cells in our body decreases, being perhaps the explanation for the failure of some organs repair during ageing. In studies with bone marrow conducted in over 1,000 patients [97–104], it was noted that the bone marrow cellularity: (a) decreases with increasing age and (b) decreases with the prevalence of connective tissue progenitors with increasing age in women. The total number of progenitors represents the product of the nucleated cells and the prevalence of progenitors in the aspirate plus a decline in the number of nucleated cells can be corrected by an increase of the volume aspirated.

Proliferation/expansion potential of hMSCs is affected by the *in vitro* culture conditions, which results in changing of cell/culture morphology. An interesting example is that under established *in vitro* conditions, hMSCs grow as a monolayer, but when cultured in hypoxic atmosphere, a condition found in many tumors *in vivo,* they continue to proliferate and the cell density increases, showing a 30-fold higher expansion rate [105]. The investigation of the proliferative potential of cells *in vitro* is necessary due to the possibility of tumor formation by some adult stem cells and especially embryonic stem cells. This process is a result of the loss of normal cellular control and is an initial aspect of cancerous tumor formation. It is important to note that most tumors arise from dividing populations of stem or precursor cells. Indeed, in the hematopoietic system each stage of stem cell to blast cell to be differentiated cell is associated with a leukemia or lymphoma [106]. The relative infrequency of transformation, however, suggests that the ability to self-renewing, surviving, proliferation, differentiation or transformation are closely regulated features. Thus, it commonly believed that the overall stem cell status in any self-renewing tissue is a dynamic balance between cell intrinsic and cell extrinsic factors. Furthermore, abnormalities in any of these stages will alter normal development or will affect cellular response to the normal aging process [91].

The cell proliferation also influences the karyotype of stem cells. In karyotypes of human mesenchymal stem cells (hMSCs), at least 30% of senescent hMSCs display trisomy of chromosome 8 [107]. hMSCs generally become polyploid (mainly tetraploid) at passage 20 becoming aneuploid afterwards [108]. During senescence, hMSCs deregulated genes were mainly found at the short arm chromosome region 4q22-q23, which inserted into immortal cells caused loss of proliferation. Senescence is also associated with upregulation of microRNAs, namely, hsa-mir-371, hsa-mir- 369-5P, hsa-mir-29c, hsa-mir-499, and hsa-let-7f, which causes a change in the methylation pattern [109]. Study with dental pulp stem cells revealed that about 70% of the cells exhibited

karyotypic abnormalities including polyploidy, aneuploidy, and ring chromosomes. The heterogeneous spectrum of abnormalities indicated a high frequency of chromosomal mutations that continuously arise upon extended culture. These findings emphasize the need for the careful analysis of the cytogenetic stability of cultured hDSCs before they can be used in clinical therapies. With respect to therapy application, special attention should be given to hMSC epigenetic changes and the appearance of senescence that could result in genomic abnormalities, during culture.

Miura et al. [110] demonstrated that murine bone-marrow-derived mesenchymal stem cells (BMMSCs), after numerous passages, obtained unlimited population doublings and proceeded to a malignant transformation state, resulting in fibrosarcoma formation *in vivo*. Transformed BMMSCs colonized to multiple organs when delivered systemically through the tail vein. Fibrosarcoma cells formed by transformed BMMSCs contained cancer progenitors, which were capable of generating colony clusters *in vitro* and fibrosarcoma *in vivo* by the second administration. The mechanism by which BMMSCs transformed to malignant cells was associated with accumulated chromosomal abnormalities, gradual elevation in telomerase activity, and increased c-myc expression [110]. Although aneuploidy has long been associated with cancer, it has recently been observed in cultured pluripotent and neuronal stem cells as well as normal neuronal progenitors and primary cells from blastocysts, showing that the tendency to generate aneuploid cells may also be a normal feature of regenerative systems [110–112].

Cell transformation (spontaneous or artificial) is the process, initiated by at least 2 genetic events (mutations), by which cells gain immortality. First studies of spontaneous transformation claimed that unlike hMSCs, only murine MSCs can spontaneously transform in culture [113]. It was concluded that *in vitro* BM-hMSC expansion and their use in therapy is completely safe. But in parallel, Rubio et al. [107] demonstrated that after 4 to 5 months of *in vitro* culture, 50% of the postsenescent adipose tissue-derived mesenchymal stem cell (AThMSC) clones can escape the proliferation crisis, resume proliferation, lose contact inhibition, and become tumor-like transformed mesenchymal stem cells. It was argued nonetheless that the susceptibility to malignant transformation is dependent on hMSC origin, and that AThMSCs derived from poor fat tissue stem cell are more prone to transformation than BM hMSCs derived from stem cell-rich BM [114]. This argument was banned when BMhMSC clones were shown to spontaneously transform as well [115]. A 2-stage model of spontaneous transformation was proposed, according to which a senescence crisis with proliferation arrest always precedes the resumption of proliferation that occurs when hMSCs undergo spontaneous transformation [116]. This model was widely accepted and challenged only by Wang et al., who argued that spontaneous transformation may already occur as early as at hMSC isolation [117]. Spontaneously transformed hMSCs (transformed mesenchymal stem cells) are morphologically distinct from early passage hMSCs and senescent hMSCs [107]. Spontaneous transformation is accompanied by distinct transcriptomic changes. Cells bypass the senescence crisis and transform by upregulation of c-myc expression, repression of p16 levels, acquisition of telomerase activity, Ink4a/Arf locus deletion, and Rb hyperphosphorilation [116]. In additional experiments the same authors were unable to confirm the *in vitro* hMSC transformation, since after a senescent phase, the hMSC culture became exhausted and the *in vitro* hMSC spontaneous transformation they described was an artifact due to cross-contamination with HT1080 cell line [118].

The biggest challenge in the use of stem cells in cellular therapy is the ability to maintain genetic integrity during long-term cultivation, as well as their ability to differentiate. By successive passages *in vitro*, the karyotype needs to be numerically and structurally intact, conferring genomic stability to the cells that are going to be used in cellular therapy. Cultured human adult stem cells are particularly susceptible to the acquisition of chromosomal anomalies because they require significant cell expansion [119–122].

6. Immunosuppressive Activity

Recently, scientists have been discussing the contribution that stem cells offer which leads to functional improvement of organ and body structures observed in experiments *in vivo*. Among the topics discussed, this contribution would come from tissue regeneration, cell fusion, and new blood vessels formation. Lately, the observation of the rapid postoperative recovery and rapid reestablishment of inflammatory condition caught the attention of scientists and led to deepening in the study of the fourth restorative of stem cells characteristic, the immunomodulator aspect, taken within cell-cell contact and paracrine contexts.

Many reports have shown MSCs to display low immunogenicity and profound immunomodulatory and anti-inflammatory capabilities *in vitro* [123–125]. Furthermore, MSCs have been used to treat several animal and patient diseases, including graft-versus-host disease [126], rheumatoid arthritis [127, 128], autoimmune encephalomyelitis [129, 130], and systemic lupus erythematosus [131]. Apart from bone-marrow-derived MSCs, MSCs from dental tissues, that is, periodontal ligament stem cell [132, 133], stem cells from apical papilla, and gingiva-derived MSCs [134] also have been demonstrated to have immunomodulatory effects while inhibiting the proliferation and function of T lymphocytes.

Among the major works, there are those which seek to understand the mechanism by which stem cells immunomodulate investigating the interaction of these cells with specific cells and isolated from the immune system. Bartholomew et al. [135] demonstrated that the MSCs isolated from baboon suppressed the proliferation of lymphocytes in a mixed lymphocyte culture (MLC) stimulated with ConA, and this suppression was dose-dependent. In human MSCs (hMSCs), the same was observed by Aggarwal and Pittenger [136]; in PHA-induced proliferation T cells, the inhibition was 50% to 60%. Rasmusson et al. [137] showed that the hMSCs inhibited the lyses promoted by cytotoxic

T lymphocytes and that these cells escaped from the lyses promoted by NK cells more efficiently than K562 lymphoma cells. When cocultivated with B cells, the MSCs did not inhibit, but neither promoted the B-cells proliferation [138].

The MSCs tend to alter soluble factors decreasing proinflammatory factors and increasing anti-inflammatory ones. Aggarwal and Pittenger [136] conducted a series of experiments with different immune system cells. Dendritic cells (DCs) type 1 cocultured with hMSCs decreased levels of lipopolysaccharide (LPS)-induced TNF-α; DCs type 2 cocultured with hMSC increased levels of IL-10 after LPS stimulus. The same results were observed with TH1 and TH2 cells. TH1 effector cells in the presence of hMSCs decrease in IFN-γ; TH2 in the presence of hMSCs increase IL-4 levels. Finally, hMSCs were cocultured with IL-2-stimulated NK cells, that resulted in a decrease in IFN-γ. The production of immunoglobulins is also affected in the presence of MSCs. It has been seen that the MSCs in contact with spleen mononuclear cells (MNCs) stimulated with LPS reduced the IgG production, and that this effect is LPS dose-dependent. With a strong LPS stimulus, the MSCs have led to a reduced production of IgG, but with a low stimulus, they have led to an increase in IgG production. With enriched B cells, there was an increase in IgG production when grown with LPS and this increased even more in the presence of MSCs. While these cocultures did not influence the levels of IL-2, they raised the levels of IL-6, in the presence or absence of LPS, an important interleukin for differentiation and production of immunoglobulin [138]. Other factors secreted in cultures with hMSCs, in addition to the IL-6, were observed such as IL-8, PGE2, and vascular endothelial growth factor (VEGF) [136].

Then, the question whether there is a molecule that can have a central influence on the whole process of immunomodulation performed by stem cells remains. Aggarwal and Pittenger [136] conducted an investigation of the involvement of PGE2 in coculture with human peripheral blood mononuclear cells (PBMCs). Using the indometacin, an inhibitor of PGE2, they observed an increase in PBMCs proliferation, the same behavior found when the PBMCs are not cocultured with MSCs. More specifically, it was noted the increase in TNF-α and IFN-γ from the activated DCs and T cells when the coculture with MSCs received PGE2 inhibitors. This gives an indication of which PGE2 is a candidate molecule capable of influencing many immunomodulators aspects. This finding is further reinforced with MSCs enhancement of PGE2 production when incubated with the proinflammatory recombinant cytokines TNF-α or IFN-γ, indicating a negative feedback stimulated by these cytokines.

Chan et al. [139] observed that the MSCs have APC (antigen-presenting cells) characteristic. In APC test, MSCs challenged with *C. albicans* and *T. toxoid* were cocultivated with activated CD4+ cells. As time went by, an increased IFN-γ concentration was observed. The authors found that the MHC II molecule has its expression decreased when the cells are in the presence of high concentrations of IFN-γ, while expression remains unchanged at low concentrations. Along with the increase in the IFN-γ concentration, a decrease in the CD4+ cells proliferation was observed. The question posed is how could MSCs act as APCs in a microenvironment of immune responses when IFN-γ levels are expected to be elevated? The authors proposed that MSCs possess the characteristic of PCA at a time limited to a period before the inflammatory response, where the concentration of IFN-γ is low. Locking the IFN-γ receptor (IFNγRI) using an anti-IFNγRI antibody, there was no expression of MHC II. At the same time, it was shown the need of the IFNγRI activation to induce APC function in MSCs, where the MSCs treated with one control isotype played the role of APC increasing CD4+ cells proliferation and MSCs treated with anti-IFNγRI did not promote the CD4+ cells proliferation. Since the expression of MHC II depends on the IFNgRI activation by IFN-γ, the MSCs' APC role is only possible moments before the IFN-γ levels increase during the immune process and once raised, the MSCs modulate to an anti-inflammatory function [140, 141]. Ryan et al. [142] reported that MSCs stimulated with IFN-γ increased the HGF and TGF-β1 production without changing the levels of IL-10. Human IL-10, TGF-β1, and HGF are known to have immunomodulatory properties. These cytokines have shown to reduce the proliferation of PBMCs in MLC test. Another molecule that suppresses the proliferation of PBMCs by MSCs stimulated with IFN-γ is the indoleamine 2,3,dioxygenase (IDO), which occurs via the accumulation of kynurenine, a metabolite of tryptophan [142].

The immunomodulatory capacity of MSCs is not only in the suppression of cell proliferation caused by an immune reaction, it extends to the change of cell types linked to anti-inflammatory processes. The cocultivation of MSCs with CD4+ cells, resulted in a CD25 and FoxP3 increased expression, molecules that characterize Treg cells. The soluble factors such as IL-10, TGF-β1, and PGE2 alone did not promote the increase of positive cells for the markers mentioned in culture of CD4+ cells alone. However, cultures of PBMCs that received these molecules showed an increase in the CD25 and FoxP3 expression, without the presence of MSCs, indicating that probably other cells of the immune system aided in induction of Tregs. The cocultivation of MSCs with CD4+ cells in the presence of TGF-β and PGE2 inhibitors has decreased the expression of FoxP3 and CD25 proteins on CD4+. This indicates that the cell-cell contact and these factors are required to induce Tregs by MSCs [143].

Aggarwal and Pittenger [136] proposed a model of MSCs interaction with various immune cells and suggested that the MSCs inhibit or limit the inflammatory response, besides promoting mitigating paths and anti-inflammatory effect. When the MSCs are present in an inflammatory environment created artificially (*in vitro*), they change the immune response by inhibiting DC1 inflammatory signaling (decreasing IL-12 and TNF-α secretion) and promoting DC2 anti-inflammatory signaling (increasing the secretion of IL-10). Furthermore, when the immature effector T cells are present, the MSCs may interact with and inhibit the development of TH1 and NK signaling (decreasing the secretion of INF-γ) and promoting TH2 anti-inflammatory signaling and Treg suppressive effect (increasing IL4 secretion). Yagi et al. [144] proposed a model for the interactions of cytokines which MSC express MHC-II and function as

APCs, at low levels of IFN-γ. At high levels of IFN-γ, MHC-II is downregulated MHC-II and B7-H1 is upregulated. IFN-γ and TNF-α individually stimulate MSCs to upregulate PGE2, COX-2, and/or IDO. These mediators can inhibit function of immune cells such as T cell, NK cell, and DC.

Other sources of stem cells have the immunomodulator aspect have been investigated. Pierdomenico et al. [145] compared MSCs with dental pulp stem cells (DPSCs) in their immunosuppressive capacity. The authors found that the DPSCs are more immunomodulatory than MSCs. Stem cells from human exfoliated deciduous teeth (SHEDs) are also shown to be more efficient in inhibiting the proliferation of Th17 cells than MSCs [146].

The inhibitory effect of the MSCs over the MLC is not dependent on the origin of the MSCs (autogenous or allogeneic), that can be stated as the most important result of this context [135, 140]. This immunomodulatory effect remains in those cells even after differentiation, as shown *in vitro*, by Le Blanc et al. [140]. MSCs which were induced into adipogenesis, chondrogenesis, and osteogenesis did not express class II MHC. The differentiated MSCs continued to inhibit the T lymphocytes proliferative response, being this feature improved when treated with INF-γ [141]. This could show that those cells may be transplanted between patients with different HLA.

As previously observed, apparently, the immunomodulatory property of the stem cells is dependent on the environment in which those are inserted. Seemingly, the stem cells have a homeostatic effect and also possess APC characteristics. During the initial part of an inflammatory process, the MSCs increase their potential of antigen presenting in order to fight infection. However, as time progresses, the pro-inflammatory environment immunomodulates the MSCs in such a way that those assume an anti-inflammatory character, this feature seems to regulate the whole pro-inflammatory medium towards homeostasis.

Recently, it has been published a creative strategy of dealing with autoimmune diseases. Zhao et al. [147] applied this strategy directly on type I diabetes patients. These authors developed an apparatus, which enclose nine plates, piled upon each other, where the umbilical cord stem cells (CB-SCs) were seeded. The patient's circulatory system was connected to a cell sorter in order to isolate lymphocytes, which after collected were transferred directly to the equipment containing the CB-SCs. After 8- to 10-hour procedure, the lymphocytes were reintroduced into the patient, in a closed circuit. The procedure's result was assessed by measuring the C-peptides levels (a product yielded during the biosynthesis of insulin, which indicates β-cells functioning). Patients treated have shown increasing levels of C-peptide and reduction in the mean values of glycated hemoglobin A1C (HbA1C). These results are promising, since the patients who would not have residual function on the β-cells began to show functional improvement, demonstrated by the glucose-stimulated C-peptide levels, even after 40 weeks past the procedure. It was also shown an increase in the Treg cells population and reestablishment of the TH1-, TH2-, and TH3-related cytokines levels. The data indicates that the immune system cells were reprogrammed to recognize the patient's tissue as its own. This statement is reinforced by the evidence that patients with no residual β-cell function had the metabolism control improved, which indicates reduction on autoimmunity and pancreatic islets recovery.

7. Conclusion

Advances in researches aiming for cellular therapy have brought important impacts on the medical field with promises of treatment of many human diseases, and in the biotechnology area with the generation of various products and biomaterials. However, as reported here, it was shown that for cell therapy success and safety, it is necessary to overcome certain limitations, such as the use of animal component in cell cultivation, for instance, the fetal bovine serum. Choosing the stem cells that will be used in cellular therapy is also very important in terms of clinical safety for the patient, since the scientific literature has been reporting the teratogenic potential of embryonic stem cells and iPSCs, and possible adult stem cell lines. In addition to the choice of stem cells, another parameter is the appropriate cell dose for a successful treatment. The cells, most commonly reported in scientific studies, are the haematopoietic origin cells. Based on these reports, we have shown that there is no consensus yet on the amount of stem cells to be transplanted and the patient's body weight. The hematopoietic stem cells are not able to be expanded *in vitro*, which does not happen with adult stem cells from nonhematopoietic origin, that can be expanded even in low passages in huge quantities, bypassing the current problem of haematopoietic cells that can only be transplanted in individuals with less than 40 kg on the basis of the quantity of CD34+ progenitors cells. Besides the cellular type and dosage, the proliferation and senescence should be investigated before transplantation because there are reports that chromosomal changes may occur during *in vitro* cultivation, and such changes could affect the cells engraftment. Therefore, the genomic stability must be considered by conducting karyotype analysis to ensure stability and normality at the occasion of transplantation, although other factors such as miRNA and chromatin changing are a challenge for the future. The use of cells which show immunosuppressive and anti-inflammatory activities is also interesting for cellular therapy, since they overcome the current compatibility problem between individuals and may reinforce the therapeutic success. Summarizing, cell therapy safety and success are bound to be achieved by the characterization of stem cells before those critical issues.

Conflict of Interests

The authors certify that they have no commercial or associative interest that represents a conflict of interests in connection with the paper.

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Review Article

Ultrastructural Evidence of Exosome Secretion by Progenitor Cells in Adult Mouse Myocardium and Adult Human Cardiospheres

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The demonstration of beneficial effects of cell therapy despite the persistence of only few transplanted cells *in vivo* suggests secreted factors may be the active component of this treatment. This so-called paracrine hypothesis is supported by observations that culture media conditioned by progenitor cells contain growth factors that mediate proangiogenic and cytoprotective effects. Cardiac progenitor cells in semi-suspension culture form spherical clusters (cardiospheres) that deliver paracrine signals to neighboring cells. A key component of paracrine secretion is exosomes, membrane vesicles that are stored intracellularly in endosomal compartments and are secreted when these structures fuse with the cell plasma membrane. Exosomes have been identified as the active component of proangiogenic effects of bone marrow $CD34^+$ stem cells in mice and the regenerative effects of embryonic mesenchymal stem cells in infarcted hearts in pigs and mice. Here, we provide electron microscopic evidence of exosome secretion by progenitor cells in mouse myocardium and human cardiospheres. Exosomes are emerging as an attractive vector of paracrine signals delivered by progenitor cells. They can be stored as an "off-the-shelf" product. As such, exosomes have the potential for circumventing many of the limitations of viable cells for therapeutic applications in regenerative medicine.

1. Introduction

Myocardial infarction and ensuing heart failure are the leading cause of mortality in Western countries. Infarction causes a massive loss of cardiomyocytes, which are replaced by scar tissue. To compensate for lost contractile cells, the remaining cardiomyocytes undergo hypertrophy and the heart remodels. These adaptive mechanisms are detrimental in the long run, eventually leading to congestive heart failure. Heart transplantation remains the ultimate treatment for chronic heart failure; however, this approach is limited by donor organ shortage, graft rejection, and the need for life-long immunosuppression.

Over the past decade, cell transplantation has been evaluated as a novel approach for heart failure. An early study reported that adult mouse bone marrow (BM) hematopoietic stem cells (HSCs) injected into infarcted mouse hearts differentiated into cardiomyocytes and improved cardiac function [1]. This report sparked widespread enthusiasm for BM cell transplantation as a potential approach for repairing broken hearts. Several subsequent studies failed to confirm the ability of HSCs to transdifferentiate into cardiomyocytes [2, 3]. However, these negative results did not prevent clinical studies of autologous BM stem cell transplantation for cardiac regeneration from being initiated [4–9]. Multiple types of stem and progenitor cells, including embryonic

stem cells (ESCs), adult skeletal myoblasts, adult BM-derived mononuclear cells, purified BM-derived subpopulations (e.g., c-kit$^+$ and CD133$^+$), and BM or adipose-derived mesenchymal stem cells (MSCs) have been evaluated as cell sources for heart cell therapy [10, 11].

Almost ten years after the initiation of randomized, controlled clinical trials of cell therapy for cardiac regeneration, it must be recognized that results have been inconsistent, and the overall improvement of cardiac function in patients after myocardial infarction (MI) has been modest [12–14]. The optimal timing of cell transfer, dose, delivery technique, the mechanism of action, patient selection, cell retention, and cell survival after transplantation are poorly understood. In addition, impaired cell functionality in old patients and in those with advanced cardiovascular disease or comorbidities limits autologous cell transplantation [15]. Hence, an unresolved paradox persists between robust benefits of cell therapy in animal models and modest effects in patients.

Transdifferentiation of adult BM cells into cardiomyocytes has not been demonstrated in humans. Originally proposed as an approach for providing injured hearts with new cells capable of restoring contractile function in scar areas, the biological rationale of cell therapy has progressively shifted toward beneficial effects mediated by transplanted cells on neighboring cells, possibly including the stimulation of endogenous regenerative mechanisms. Increasing evidence suggests cell therapy, whether myoblast or BM cell-based, may act by increasing the cellular mass in the infarcted area, thereby reducing ventricular remodeling, and by preventing cardiomyocytes in the ischemic peri-infarct zone from dying. The second mechanism, which involves secreted factors, has been referred to as the "paracrine hypothesis." Growth factors and cytokines secreted by transplanted cells activate endogenous intracellular signaling pathways potentially resulting in improved survival of endogenous cells and formation of new blood vessels [16–18].

2. Adult Cardiac Stem Cells (CSCs) Expressing Stem Cell Surface Markers

The adult myocardium is a highly organized tissue comprised of multiple cell types, including cardiomyocytes, endothelial cells, vascular smooth muscle cells, multiple types of interstitial cells, and extracellular matrix (ECM), which form a cardiovascular unit [19]. Interstitial cells include cardiac-resident stem and progenitor cells (CSCs) located within stem cell niches [20]. These niches contain particular ECM components, supporting cells, nerves, and blood vessels. Several groups have isolated and characterized *bona fide* adult CSCs from rodents and humans [21–29]. Molecular markers that have been used to identify CSC populations include cell-surface epitopes expressed on stem cells in other tissues, particularly on HSCs, such as c-kit (CD117; the receptor for stem cell factor) and stem cell antigen-1 (Sca-1). At minimum, five seemingly different populations of CSCs have been described [30]. Whether these populations represent distinct entities of CSCs or different developmental stages or activation states of a single entity of CSC remains unclear. Likewise, the origin of CSCs has not been definitively established. In this regard, we and others have shown that BM-derived cells can acquire stem cell properties in the damaged heart [31, 32].

It has been speculated that cells derived from the heart itself may be a logical candidate cell source for repairing this organ, as these cells might be intrinsically programmed to support cardiac cell survival and function. In animal models of MI, injection of *in vitro* expanded CSCs has been associated with functional improvement [21–27]. However, direct comparisons of CSCs with other cell sources are needed in order to identify the most effective cell type. Recently, novel approaches based on adult cell reprogramming (induced pluripotent stem cells; iPS) have been developed [33]. Although extremely promising, these strategies are still associated with potential safety concerns. The discussion of these modalities is beyond the scope of the present work (the interested reader is referred to an excellent review published elsewhere [34]).

3. Cardiospheres (CSs) and CS-Derived Cells (CDCs)

First described in neural stem cells (neurospheres) [35], "spheres" have been considered—or named, at least—a feature of stemness. However, spheres can result not only from cell proliferation but also from cell aggregation, and therefore be clonal or nonclonal, respectively. The proportion of clonal spheres increases with decreasing cell density in a culture dish [36].

First described by Messina et al. [37], "cardiospheres" (CSs) are self-assembling spherical clusters of cells obtained by outgrowth from cardiac explants in the primary *ex vivo* tissue culture. CSs grow in semisuspension culture on poly-D-lysine. They represent the best *in vitro* model of CSC niche-like environment [38]. While undifferentiated cells proliferate in the core of the CS, cardiac-committed cells grow on the periphery. We have generated CSs from the cellular outgrowth from adult human atrial appendage explants (Figure 1). The cellular outgrowth expressed MSC surface markers (CD13$^+$, CD73$^+$, and CD105$^+$) but not hematopoietic markers (CD45$^-$). In line with previous studies [25, 37], we have demonstrated the expression of cardiac-specific genes, such as troponin I, in human CSs (Figure 2). CSs placed in a new culture dish disassembled and gave rise to a monolayer of CS-derived cells (CDCs) that formed second-generation CSs. CDCs could be expanded as monolayers on fibronectin (Figure 1). Previous studies [25, 39] reported that CDCs are clonogenic and have multilineage differentiation potential. By contrast, a recent study in rodents questioned the notion of CSs as a source of stem cells with cardiomyogenic potential [40]. This study suggested spontaneously beating CSs may result from remnants of myocardial tissue in the cellular outgrowth from cardiac explants. Regardless of this issue, CDCs have been shown to improve left ventricular ejection fraction (LVEF) in SCID beige mice 3 weeks after MI when compared to mice injected with vehicle or with adult normal human dermal fibroblasts [25]. These beneficial effects were associated with increased blood vessel formation and decreased apoptosis [41].

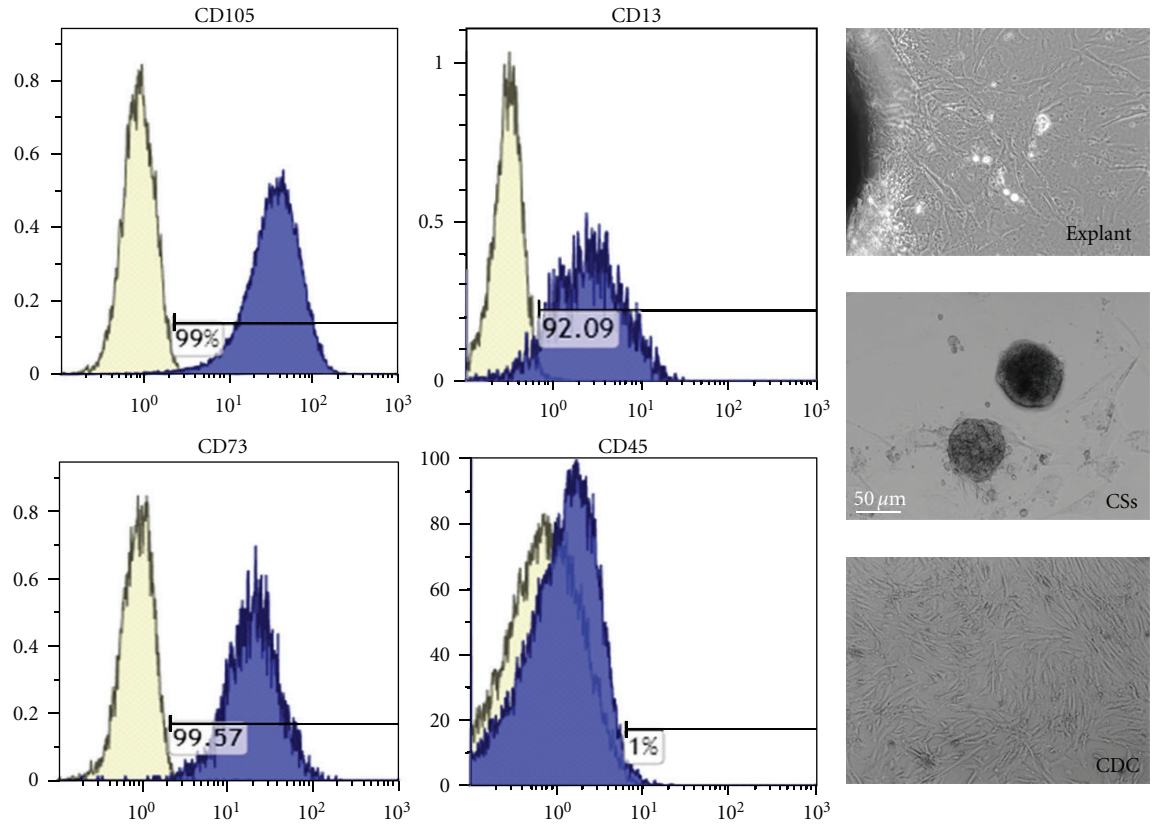


Figure 1: Left and middle panels: Flow cytometric analysis of the cellular outgrowth from a human atrial appendage explant in the primary tissue culture, showing MSC ($CD13^+$, $CD73^+$, and $CD105^+$) but not hematopoietic ($CD45^-$) marker expression. Upper right panel: Photomicrograph of a human atrial explant with cellular outgrowth; middle right panel: human CSs; lower right panel: human CDCs.

Autologous c-kit^+, differentiation lineage-negative (Lin^-) CSCs, as well as CDCs, have recently been tested in initial clinical studies. These two clinical trials of CSC therapy for cardiac repair are briefly discussed in the next section.

4. Clinical Studies of Autologous CSCs for Ischemic Heart Disease

The first clinical trial of autologous CSCs for ischemic heart disease was the stem cell infusion in patients with ischemic cardiomyopathy (SCIPIO) trial (registered with ClinicalTrials.gov, number NCT00474461) [42]. This phase-1 clinical trial tested autologous c-kit^+/Lin^- CSCs for treatment of heart failure resulting from ischemic heart disease. In stage A of this trial, patients with reduced LVEF (≤40%) after MI before coronary artery bypass grafting (CABG) were consecutively enrolled in the treatment and control groups. In stage B, patients were randomly assigned to the treatment or control group. Autologous CSCs were administered by intracoronary infusion at a mean of 113 days (SE 4) after surgery. Sixteen patients were assigned to the treatment group and seven to the control group. No cell therapy-related adverse effects were reported. In 14 CSC-treated patients who were analyzed, LVEF increased from 30.3% (SE 1.9) before CSC infusion to 38.5% (SE 2.8) at 4 months after infusion ($P = 0.001$). Conversely, in seven control patients LVEF did not change during the corresponding time interval (30.1% [SE 2.4] at 4 months after CABG versus 30.2% [SE 2.5] at 8 months after CABG). In eight treated patients studied at 1 year, LVEF increased by 12.3 LVEF units [SE 2.1] versus baseline ($P = 0.0007$). These results suggest intracoronary infusion of autologous c-kit^+/Lin^- CSCs may enhance LV systolic function in patients with heart failure after MI. In the seven treated patients in whom cardiac magnetic resonance imaging (MRI) was done, infarct size decreased from 32.6 g (SE 6.3) by 7.8 g (SE 1.7; 24%) at 4 months ($P = 0.004$) and 9.8 g (SE 3.5; 30%) at 1 year ($P = 0.04$). This reduction in scar is difficult to interpret due to the lack of MRI data in the control group.

The prospective, randomized CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction (CADUCEUS) trial (registered with ClinicalTrials.gov, NCT00893360) evaluated autologous CDCs in patients with reduced LVEF (=25–45%; mean baseline value =39%; [SD 12]) 2–4 weeks after AMI [43]. Seventeen patients received CDCs and eight patients were randomized to the control group. Autologous CDCs grown from endomyocardial biopsy specimens were infused into the infarct-related artery 1.5–3 months after MI. Biopsy samples yielded the prescribed cell doses within 36 days (SD 6). By 6 months, no patients had died, developed cardiac tumors, or major

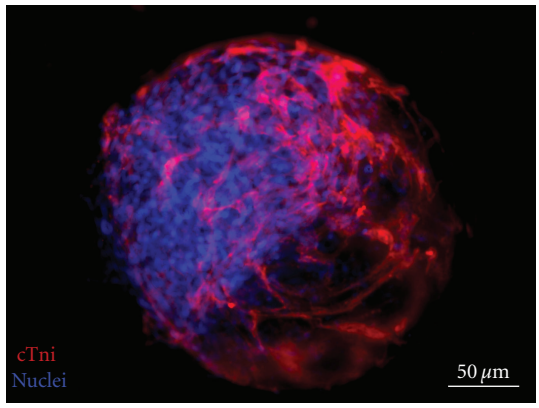


Figure 2: Immunostaining of a human CS showing expression of cardiac troponin I (red); nuclei stain blue.

adverse cardiac events in either group. Four patients (24%) in the CDC group had serious adverse events compared with one control (13%; $P = 1.00$). MRI analysis showed reductions in scar mass by 28% by 6 months and 42% by 12 months ($P = 0.001$), increases in viable heart mass by 13.0 g at 6 months ($P = 0.01$), greater regional contractility (-11.8% versus -8.5%; $P = 0.02$), and regional systolic wall thickening ($P = 0.015$) in the CDC group compared with controls. Changes in end-diastolic volume, end-systolic volume, and LVEF did not differ between groups by 6 months. These results suggest that intracoronary infusion of autologous c-kit$^+$/Lin$^-$ CSCs or CDCs after myocardial infarction is safe and might be beneficial. Larger clinical trials to test the efficacy of these approaches are being planned.

5. Paracrine Effects of Transplanted Cells

Based on the number of human-specific cells relative to overall increases in capillary density and myocardial viability, Chimenti et al. [41] estimated that direct progenitor cell differentiation quantitatively accounted for 20% to 50% of the observed effects of human CDCs transplanted into infarcted SCID mouse hearts. Conversely, a large part of these effects seemed attributable to endogenous cells. *In vitro*, culture media conditioned by human adult CDCs prevented apoptosis in neonatal rat ventricular myocytes under hypoxic conditions, while promoting angiogenesis from human umbilical vein endothelial cells (HUVECs). *In vivo*, human CDCs secreted hepatocyte growth factor-1 (HGF-1), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF) when transplanted into the same SCID mouse model of MI, where they were shown to induce tissue regeneration and improve function. Injection of CDCs into the peri-infarct zone increased the expression of the prosurvival factor Akt, reduced apoptosis, and increased capillary density. Although myocardial perfusion was not directly measured in this study, increased capillary density was consistent with a role for angiogenesis in functional improvement. These findings were interpreted as evidence for paracrine effects of CDCs exceeding those of direct regeneration. In another study, Tang et al. [44] reported beneficial effects of paracrine factors secreted by rat c-kit$^+$ CSCs delivered by intracoronary infusion 4 weeks after MI. Although no engrafted donor cells were found in some hearts in the cell therapy group, scar area was reduced and cardiac output increased compared with the control group. The number of cardiomyocytes, blood vessels, and endogenous cardiac progenitor cells was increased in hearts injected with c-kit$^+$ CSCs, even in the case when no engrafted cells were observed. Smits et al. [45] showed that injection of human cardiac progenitor cells into infarcted mouse hearts reduced cardiac remodeling 3 months after MI, despite the fact that only 3-4% of the injected cells could be found in the hearts at this time point. Together, these findings suggest factors released by CSCs may mediate sustained beneficial effects, including angiogenesis and improved cardiomyocyte survival. In this regard, CDCs have been shown to secrete a number of growth factors [41] and microRNAs (miRNAs) [46] that regulate intracellular signaling pathways in neighboring cells. Culture media conditioned by ESCs or MSCs were shown to improve myocardial function after ischemia by reducing apoptosis and infarct size both *in vitro* and *in vivo* [47–49]. Additional types of interstitial cells including resident cardiac immune cells [50] may also contribute to the secretion of cytokines and growth factors into their microenvironment. Collectively, these studies indicate that paracrine effects of progenitor cells are a central mechanism of cell therapy. This notion implies a dispensable role for cell transplantation in therapeutic approaches for cardiac regeneration [17].

6. Microparticles and Exosomes

The demonstration of beneficial effects of cell therapy despite short-lived survival of the delivered cells, along with the observed trophic effects of culture media conditioned by progenitor cells, suggests that secreted factors may be the active component of cell therapy for cardiac regeneration, as mentioned. Cells communicate with each other via released molecules such as short peptides, proteins, nucleotides, and lipids that bind to surface receptors on neighboring cells. In addition, eukaryotic cells communicate with each other through the release of microparticles and exosomes in their extracellular environment. Microparticles are

a heterogeneous population of spherical structures with a diameter of 100–1000 nm, which are released by budding of the plasma membrane (ectocytosis) as phospholipid vesicles that express antigens specific of their parental cells [51]. Circulating microparticles are increased in a number of disease conditions, such as inflammatory and autoimmune diseases, atherosclerosis, and cancer. Distinct from microparticles, exosomes are membrane vesicles with a diameter of 40–100 nm, formed by endocytosis, a process that involves the sequestration of plasma membrane proteins within the exosomes. Exosomes are stored intracellularly in endosomal compartments and are secreted when these multivesicular structures fuse with the cell plasma membrane [52–55].

Exosomes display a broad spectrum of bioactive substances on their surface and carry a concentrated set of proteins, lipids, and even nucleic acids that are taken up by other cells and regulate their function [54–56]. Some exosomal membrane proteins are cleaved by proteases, and the resulting fragments may act as ligands for cell surface receptors in the target cell. Exosomes can also transfer exosomal proteins and RNA nonselectively by fusing with target cells. They are released by many cell types, including dendritic cells, mast cells, B and T cells, platelets, neurons, tumor cells, and MSCs.

Exosomes do not carry a random array of the intracellular proteins but a specific set of proteins derived from the plasma membrane, endocytic pathway, and the cytosol, with only low amounts of proteins from other intracellular compartments [53, 56]. Most exosomes contain tetraspanins (CD81, CD63, and CD9) which play important roles in cell penetration, invasion and fusion events, as well as multivesicular body molecules (Alix, Tsg101, and clathrin), heat shock protein 70 (HSP70), and Rabs that regulate exosome docking and membrane fusion [57]. Exosomes also contain annexins, metabolic enzymes, ribosomal proteins, signal transduction molecules, adhesion molecules, ATPases, cytoskeletal and ubiquitin molecules, growth factors, cytokines, mRNA, and microRNA (miRNA) molecules [53, 56, 58]. An exosome protein and RNA database (ExoCarta) is available at http://exocarta.ludwig.edu.au/. In addition to molecules shared by exosomes from multiple cell types, exosomes carry specific proteins from their parental cell type. It has been shown that mRNAs carried by exosomes can be translated into proteins in the target cell, indicating that exosomes can act as a vector of genetic information. ESC-derived microvesicles have been shown to reprogram hematopoietic progenitors by mRNA transfer and protein delivery [59]. miRNA families can be selectively secreted into the extracellular environment via exosomes [60]. Dendritic cells release exosomes that are loaded with distinct sets of miRNA dependent on the status of dendritic cell activation [61]. These exosomes fuse with target cells, thereby transferring miRNAs that can repress mRNAs in these cells. Thus, exosomes can mediate epigenetic effects by transferring specific miRNA molecules between cells.

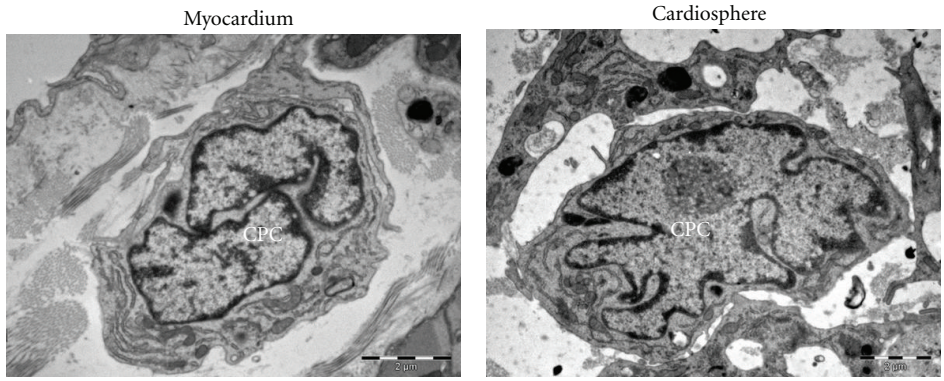
Biological effects of exosomes are cell type-specific, reflecting their molecular composition. Recently, Sahoo et al. [62] reported angiogenic effects of exosomes derived from human $CD34^+$ BM stem cells in isolated endothelial cells and in murine models of vessel growth. These exosomes were enriched with proangiogenic miRNAs. By contrast, exosomes derived from CD34-depleted BM cells lacked angiogenic activities. In some of the *in vitro* and *in vivo* assays, the exosomes from $CD34^+$ cells appeared more potent than the cells themselves, possibly as a result of the durability of the exosome in culture. Vrijsen et al. [63] reported that exosomes mediated the angiogenic activity of media conditioned by human fetal cardiac progenitor cells *in vitro*. Timmers et al. [49] showed that injection of conditioned medium from ESC-derived MSCs reduced infarct size and improved cardiac function in a pig model of ischemia/reperfusion, as mentioned above. Exosomes within the conditioned medium were shown to contain the active component [64]. Lai et al. [65] found that exosomes secreted by MSCs similarly reduced myocardial ischemia/reperfusion injury in mice.

In extracardiac biological systems, exosomes were shown to secrete synuclein that impacted neuronal survival [66], or αB crystallin from polarized human retinal pigment epithelium, which provided neuroprotection to adjacent cells [67], just to mention a few examples. Altogether, exosomes act as vectors for the intercellular exchange of biological signals and information, which mediate cell activation, phenotypic changes, and reprogramming of cell function. Exosomes and microparticles represent a transcellular delivery system that expands the limited transcriptome and proteome of recipient cells and establishes a communication network among cells [51].

7. Ultrastructural Evidence of Exosome Secretion by Cardiac Progenitor Cells

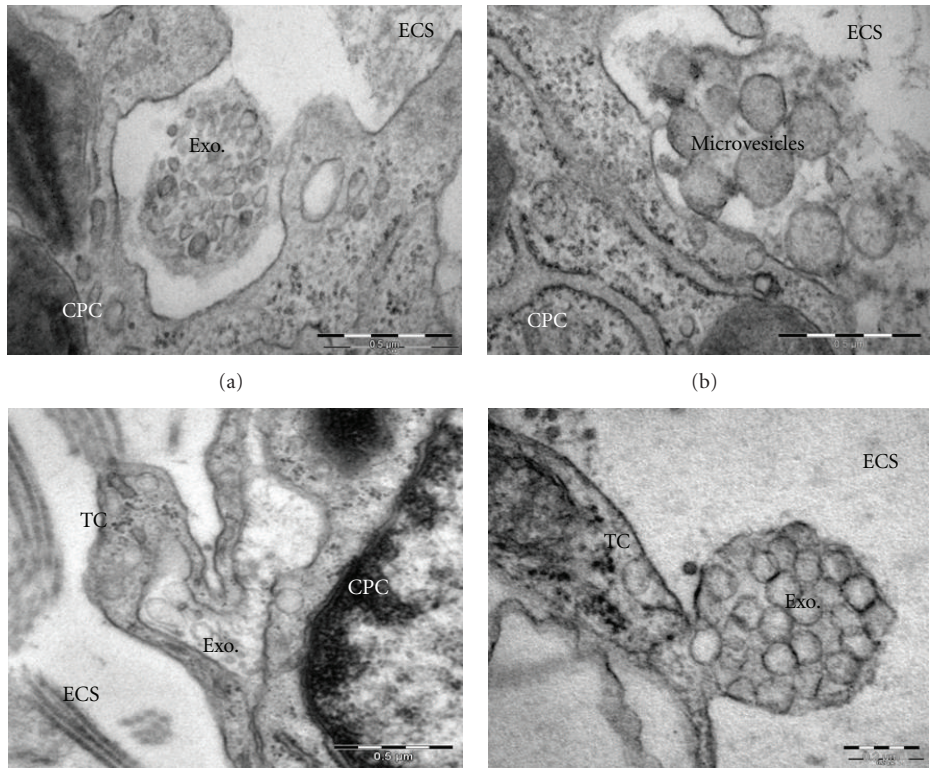
Since CDCs deliver beneficial paracrine signals to injured myocardium [41], and since exosomes have been identified as the active component of the paracrine effects of $CD34^+$ HSCs [62] or MSCs [64, 65] in infarcted hearts, we investigated whether CSCs and human CSs secrete exosomes. Figure 3 shows transmission electron micrographs of progenitor cells within a stem cell niche in a mouse adult heart, as well as in an adult human CS *in vitro*. Progenitor cells exhibit a similar ultrastructural appearance in the two contexts. Here, we also provide, for the first time, ultrastructural evidence of exosome and microvesicle secretion by progenitor cells in mouse adult heart and in human CSs (Figure 4). Microvesicles ($\approx$200 nm diameter) have an electron-dense content. Exosomes can be distinguished from microvesicles, apoptotic bodies, and other types of membrane vesicles by size and ultrastructural appearance. Exosomes and microvesicles seems to be an important mechanism involved in the heterocellular communication in the adult heart [68], especially between telocytes [69] and resident progenitor cells [68, 70]. Some of us recently showed that exosomes emerge from telocytes in the border zone of myocardial infarction [71], consistent with a potential role for this secreted component in neoangiogenesis; however, a quantitative analysis was not performed.

Transmission electron micrographs suggesting exosome uptake by cardiomyocytes in the adult mouse heart are



(a) (b)

Figure 3: Transmission electron micrographs showing a cardiac progenitor cell (CPC), characterized by a large nucleus and a thin cytoplasmic rim, in adult mouse myocardium (a) and in an adult human CS *in vitro* (b).



(a) (b)

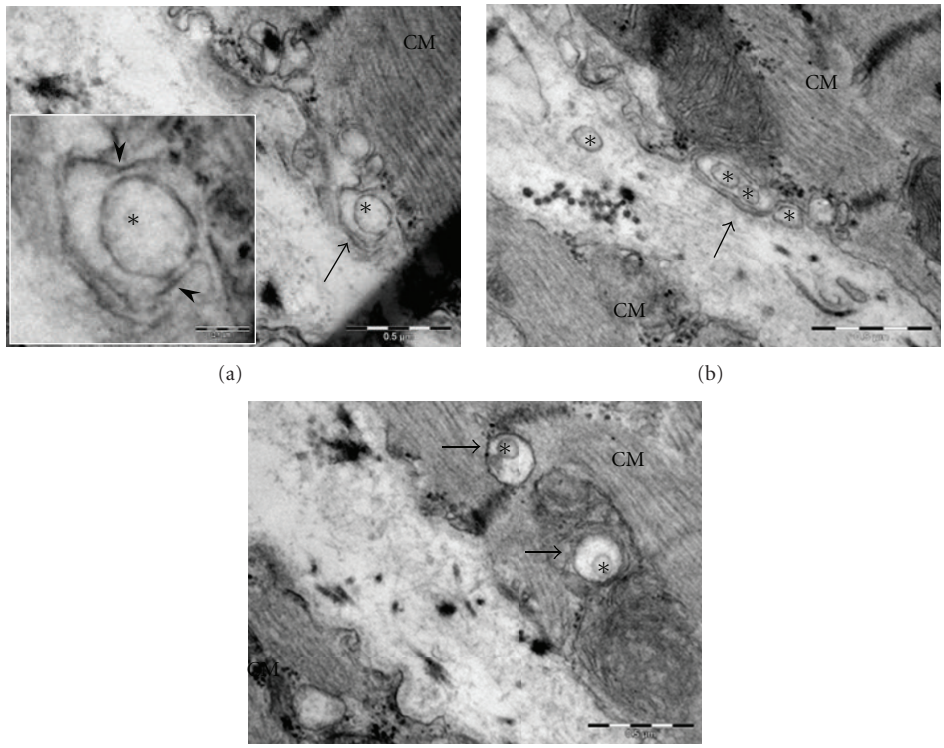
(c) (d)

Figure 4: Transmission electron micrographs showing exosomes (≈75 nm diameter; Exo. (a)) and microvesicles (≈200 nm diameter; (b)) secreted by cardiac progenitor cells (CPC) in a human CS *in vitro*. Exosomes in adult mouse myocardium ((c)-(d); TC: telocyte; ESC: extracellular space).

shown in Figure 5. Cardiomyocytes seem to encircle exosomes on their cell surfaces with thin cytoplasmic processes, and exosomes are then incorporated into cardiomyocytes. However, this preliminary observation needs to be analysed in a larger number of samples. The molecular content and functional activities of exosomes secreted by human CSCs and CSs also remain to be characterized. An additional, unanswered question relates to changes in exosome secretion under normal and ischemic conditions.

8. Therapeutic Potential of Exosomes

Exosomes may circumvent many of the hurdles associated with the use of replicating cells as a therapeutic agent, such as the risk that replicating cells may increase in an uncontrolled manner over time or exert persisting biological activities regardless of the clinical needs of the patient. Also, many autologous stem cell therapies require *ex vivo* cell expansion, and therefore the preparation of the therapeutic



(a) (b)

(c)

Figure 5: Transmission electron micrographs showing exosomes in an adult mouse heart. (a) Exosomes (asterisks) located at the cell surface of a cardiomyocyte (CM). The latter extends thin cytoplasmic processes (arrow) that encircle the exosome. Inset: higher magnification view showing dense nanostructures (arrowheads) at the interface of the CM membrane and the membrane of the exosome, suggesting involvement of molecular interactions in exosome uptake by CMs. (b) Exosomes (asterisks) encircled by cytoplasmic processes (arrow). (c) Exosomes (asterisks) taken up by the CM within small, round cytoplasmic structures (arrows).

product may take several weeks. Moreover, autologous cells from aged patients have limited regenerative potential [15], whereas allogeneic cells are generally rejected by the immune system (with the possible exception of MSCs, which mediate immunomodulatory effects [72]). A hypothetical advantage of exosomes is that it might be possible to use allogeneic cells from young, healthy individuals for their production, although this remains to be demonstrated. Exosomes are supposedly less immunogenic than their parental cells owing to a lower content of membrane-derived proteins including major histocompatibility complex (MHC) molecules. However, this may depend on the parental cell type. Since exosomes secreted by dendritic cells can transfer MHC class II molecules and stimulate immune cells [73], they have been tested in clinical trials in cancer patients [74–76]. CDCs have been shown to express MHC class I but not class II molecules [77], and therefore exosomes secreted by CDCs can be assumed to lack MHC class II. If alloimmunogenicity of exosomes secreted by CSs or CDCs turns out to be negligible, it will be possible to develop "off-the-shelf" therapies based on exosomes from young, healthy donors. Obviously, cell-free products offer a number of advantages compared with cell transplantation. Cell-free products can be standardized and tested in terms of dose and biological activity. Exosomes could be stored without potentially toxic cryopreservatives at -20°C for 6 months with no loss in their biochemical activities [64]. Importantly, exosomes have been shown to protect their contents from degradation *in vivo* [58, 78], thereby potentially preventing some of the problems associated with small soluble molecules such as cytokines, growth factors, transcription factors, and RNAs, which are rapidly degraded. The durability of the exosomes in culture permits to achieve high doses of exosomes through collection from culture medium in which exosomes are secreted over periods of time [62]. Scalable systems for exosome production to support large-scale, commercially viable manufacturing processes have been described [64]. Exosomes therefore exhibit several attractive features as a therapeutic agent. On the other hand, potential limitations should be considered as well. For example, exosomes contain a mixture of biologically active molecules, some of which seem to have beneficial effects, whereas others might have detrimental (e.g., proinflammatory) effects under certain conditions. Whether exosomes will be superior to angiogenic drugs or purified, recombinant growth factors and other peptides within the context of cell-free approaches for tissue regeneration remains to be seen.

9. Conclusions

CSs represent an *in vitro* model that recapitulates several aspects of cell-cell interactions between CSCs and other

cells in the heart. Exosomes may be a key mechanism by which cardiac progenitors communicate with each other and deliver paracrine signals to neighboring cells. Here, we provide, for the first time, ultrastructural evidence of exosome secretion by progenitor cells in the adult mouse heart, as well as in human CSs *in vitro*. Earlier studies have demonstrated beneficial effects of exosomes secreted by HSCs and MSCs in animal models of MI. These studies suggest exosomes may offer major advantages as a cell-free therapeutic product for cardiac regeneration.

Authors' Contribution

L. Barile and M. Gherghiceanu contributed equally to this work.

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